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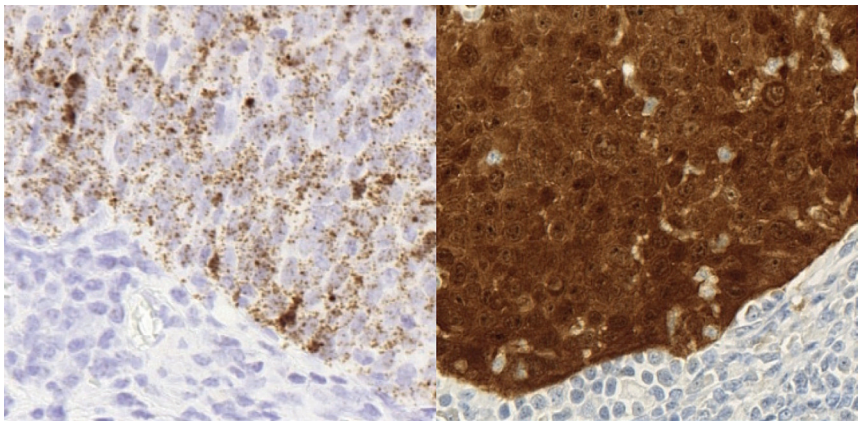
TIMO CARPÉN

NOVEL DIAGNOSTIC AND PROGNOSTIC ASPECTS OF HPV-RELATED AND -UNRELATED OROPHARYNGEAL CANCER



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NOVEL DIAGNOSTIC AND PROGNOSTIC ASPECTS OF HPV-RELATED AND -UNRELATED OROPHARYNGEAL CANCER

Timo Carpén

ACADEMIC DISSERTATION

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To Juulia, Siiri and
Aarni

Life is not a problem to be solved,
but a reality to be experienced.
Soren Kierkegaard

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1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Randén-Brady R*, Carpén T*, Jouhi L, Syrjänen S, Haglund C, Tarkkanen J, Remes S, Mäkitie A, Mattila P S, Silén S* and Hagström J*. In situ hybridization for high risk HPV E6/E7 mRNA is a superior method for detecting transcriptionally active HPV in oropharyngeal cancer. Hum Pathol. 2019;90(8):97-105. [Epub ahead of print] *Equal contribution

II Carpén T, Sjöblom A, Lundberg M, Haglund C, Markkola A, Syrjänen S, Tarkkanen J, Mäkitie A, Hagström J, and Mattila P S. Presenting symptoms and clinical findings in HPV-positive and HPV-negative oropharyngeal cancer patients. Acta Otolaryngol. 2018;138(5):513-158.

III Carpén T, Syrjänen S, Jouhi L, Haglund C, Randén-Brady R, Mäkitie A Mattila P S, and Hagström J. Epstein-Barr virus (EBV) and polyomaviruses are detectable in oropharyngeal cancer and EBV may have prognostic impact. (Submitted)

IV Carpén T, Saarilahti K, Haglund C, Markkola A, Tarkkanen J, Hagström J, Mattila P S and Mäkitie A. Tumor volume as a prognostic marker in p16-positive and p16-negative oropharyngeal cancer patients treated with definitive intensity-modulated radiotherapy. Strahlenther Onkol. 2018;194(8):759-770.

V Carpén T, Sorsa T, Jouhi L, Tervahartiala T, Haglund C, Syrjänen S, Tarkkanen J, Hesham M, Mäkitie A, Hagström J and Mattila P S. High levels of tissue inhibitor of metalloproteinase-1 (TIMP-1) in the serum are associated with poor prognosis in HPV-negative oropharyngeal squamous cell cancer. Cancer Immunol Immunother. 2019;68(8):1263-1272. [Epub ahead of print]

The publications are referred to in the text by their roman numerals. These original publications have been reprinted with the permission of the copyright holders.

2. ABBREVIATIONS

AJCC	American Joint Committee on Cancer
BIRC2	baculoviral IAP repeat containing 2
BKV	BK virus
CASP8	caspase 8, apoptosis-related cysteine peptidase
CCND1	Cell Cycle Regulators Cyclin 1
CD	cluster of differentiation
CDK	cyclin-dependent kinase
cD1	cyclin D1
CDKN2A	cyclin-dependent kinase inhibitor A2
CI	confidence interval
CRT	chemoradiotherapy
CT	computer tomography
DAB	diaminobenzidine
DapB	dihybrodipicolinate reductase
DFS	disease-free survival
EBER	Ebstein-Barr virus-encoded small RNA
EBV	Ebstein-Barr virus
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
FAK	focal adhesion kinase
FADD	fas associated via death domain
FFPE	paraffin-embedded formalin-fixed
Gy	gray
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
HR	high risk
HRAS	Harvey rat viral oncogene homolog
HS-PPIB	peptidylpropyl isomerase B
IFMA	immunofluorometric assay
IHC	immunohistochemistry
IMRT	intensity-modulated radiotherapy
ISH	in situ hybridization
JCV	John Cunningham virus
LMP	latent membrane protein
LR	low risk
LRC	locoregional control
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
nGTV	nodal gross tumor volume
ND	neck dissection
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B-cells

NPC	nasopharyngeal carcinoma
OPSCC	oropharyngeal squamous cell carcinoma
OS	overall survival
p16	$p16^{INK4A}$
PD1	programmed death-1
pRb	retinoblastoma protein
PCR	polymerase chain reaction
p53	protein 53
pGTV	primary gross tumor volume
PET	positron emission tomography
PIK3CA	phosphoinositide-3-kinase, catalytic, alpha polypeptide
PM	patient material
PNA	peptid nucleid acid
PT	proton therapy
qRT-PCR	quantitative reverse transcriptase-PCR
ROC	receiver operating characteristic
RT	radiotherapy
SCC	squamous cell carcinoma
SV40	Simian virus 40
TIMP	tissue inhibitor of matrix metalloproteinase
TMA	tissue microarray
TNM	tumor node metastasis
TORS	transoral robotic surgery
TP53	tumor protein 53
TRAF3	TNF Receptor Associated Factor 3
UICC	the Union for International Cancer Control
YAP1	yes-associated protein 1
3D	three-dimensional

3. ABSTRACT

Head and neck squamous cell carcinoma (HNSCC) is the 7th most common type of cancer worldwide and is mainly attributed to tobacco smoking and heavy alcohol use. The incidence of oropharyngeal squamous cell carcinoma (OPSCC) has been rapidly increasing in recent decades. The main driver behind this phenomenon is high-risk (HR) human papillomavirus (HPV) that currently comprises more than a half of new OPSCCs in numerous Western countries. HPV-positive OPSCC differs distinctly in genetic and pathophysiologic profiles and in patient outcomes from their HPV-negative counterparts. Both HPV-positive and HPV-negative OPSCC are typically diagnosed at an advanced stage and thus require multimodal treatment approaches that often impair quality of life. In addition, the prognosis of HPV-negative OPSCC has remained poor regardless of improvements in treatment strategies. The recognition of early clinical signs and reliable diagnostic tools are essential to achieve earlier and accurate diagnosis, respectively.

Both improvements in diagnostics and new prognostic markers are necessary to develop novel treatment strategies and therefore improve patient outcomes and avoid a reduction in quality of life. Due to the indisputable role of HPV in OPSCC, interest in other oncogenic viruses in HNSCC (such as Epstein-Barr virus [EBV] and polyomaviruses) has arisen. However, knowledge about the role of other oncogenic viruses in OPSCC is scarce and their prognostic role is unknown. Other clinical and biomolecular markers, such as tumor volume and matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), have shown potential prognostic impact on HNSCC; their roles in HPV-positive and HPV-negative OPSCC have however remained unclear.

Aims of the study

The first part of this thesis is focused on diagnostics in OPSCC (Studies I-III). We aimed to compare different HPV-detection methods against the standard method (i.e. p16INK4A [p16] immunohistochemistry [IHC]) to increase the accuracy of detecting active HPV from OPSCC samples (Study I). In addition, we wanted to better understand the clinical behavior of OPSCC and the role of other viruses. We evaluated the presenting symptoms and clinical features of patients with HPV-positive and HPV-negative OPSCC and investigated the presence of EBV and polyomaviruses (Studies II-III).

The second part of this thesis included an evaluation of potential prognostic markers in OPSCC (Studies III-V). We wanted to examine whether EBV and polyomaviruses are associated with patient survival in OPSCC (Study III). Additionally, we wanted to investigate the potential prognostic value of

primary gross tumor volume (pGTV), nodal (n)GTV, MMP-8, and TIMP-1 in HPV-positive and HPV-negative OPSCC patients (Studies IV-V).

Materials and Methods

The patient material of all studies consisted of consecutive OPSCC patients diagnosed between 2012 and 2016 at the Helsinki University Hospital. In addition, Study I included a consecutive OPSCC patient cohort covering years 2000 to 2009. Clinical and tumor-related factors were collected from hospital registries. Depending on the study, HPV was detected from tumor tissue samples by different methods, including p16 IHC, DNA polymerase chain reaction (PCR), and DNA and mRNA in situ hybridization (ISH). EBV and polyomaviruses were detected by PCR and EBV early transcripts (EBER) by ISH (Study III). In Study IV, pre-treatment pGTV and nGTV were evaluated and the patients were subsequrently treated with definitive intensity-modulated radiotherapy (IMRT) with or without chemotherapy. For the Study V, MMP-8 and TIMP-1 serum levels were analyzed from prospectively collected blood samples and their tissue expression was detected from OPSCC samples.

Results

ISH for HR HPV E6/E7 mRNA was found to be the most accurate method to detect transcriptionally active HPV in OPSCC (Study I). The majority of patients with HPV-positive OPSCC had a neck lump as the presenting symptom. Any sort of pain in the HN area was presented as the initial symptom among HPV-negative OPSCC patients (Study II). In addition, significant differences in other clinicopathological factors were found between these two cancer subgroups; HPV was the predominant virus in OPSCC as over a half of all tumors were HPV-positive (Study II-III). Polyomaviruses were detectable in OPSCC samples but did not impact prognosis. Instead, EBER was expressed in stromal lymphocytes adjacent to the tumor and correlated significantly with HPV (Study III). Patients with EBER-positive but HPV-negative OPSCC had poorer survival than those with HPV-negative/EBER-negative tumors and was significantly poorer when compared with HPV-positive OPSCC (Study III). Higher nGTV was significantly associated with poorer survival in p16-positive and p16-negative OPSCC patients. Additionally, pGTV had a significant impact on prognosis among p16-negative patients. The prognostic accuracy of pGTV and nGTV were better when compared with the most recent tumor-node-metastasis (TNM) classification (Study IV). In Study V, high TIMP-1 serum levels were associated with poorer survival among patients with HPV-negative OPSCC. MMP-8 was not associated with patient outcome.

Conclusions

ISH for high-risk HPV E6/E7 mRNA was found to be a superior method to detect transcriptionally active HPV in OPSCC. As the p16 protein may be overexpressed without the presence of HPV, all p16 IHC-positive OPSCCs should be considered for retesting by mRNA ISH to improve diagnostic accuracy and therefore to distribute HPV-positive and HPV-negative OPSCC patients accordingly. HPV is the predominant virus in OPSCC and the only virus that clearly stratifies patients into two different disease entities by clinical behavior and prognosis. However, HPV may not be the only viral factor related to OPSCC. EBV, or more precisely EBER, was found to correlate significantly with HPV and its presence was associated with poorer prognosis among HPV-negative patients. Tumor volume and TIMP-1 serum levels may serve as independent prognostic factors in OPSCC, especially for patients with HPV-negative OPSCC that typically have a poor prognosis.

4. SUMMARY IN FINNISH

Pään ja kaulan alueen levyepiteelisyöpä on maailmanlaajuisesti seitsemänneksi yleisin syöpätyyppi ja sen syntyminen on yhdistetty pääosin tupakointiin ja runsaaseen alkoholin käyttöön. Suunielun levyepiteelisyövän ilmaantuvuus on nopeasti lisääntynyt viimeisen muutaman vuosikymmenen aikana. Merkittävin tekijä tämän ilmiön taustalla on ihmisen korkean riskin papilloomavirus (HPV), joka nykypäivänä liittyy yli puoleen tuoreista suunielusyöpätapauksista useissa länsimaissa. HPV-positiivinen suunielusyöpä eroaa merkittävästi geneettiseltä ja patofysiologiselta profiililtaan sekä ennusteeltaan HPV-negatiivisesta suunielusyövästä. Suunielusyöpä diagnosoidaan usein vasta taudin myöhäisessä vaiheessa riippumatta HPV:n olemassaolosta vaatien laajoja hoitomenetelmiä, jotka usein heikentävät elämänlaatua. Lisäksi HPV-negatiivisilla potilailla ennuste on pysynyt huonona riippumatta kehittyneistä hoitomenetelmistä. Taudin varhaisten kliinisten merkkien tunnistaminen ja luotettava diagnostiikka ovat ensiarvoisen tärkeitä tarkan ja aikaisemman diagnoosin saavuttamiseksi.

Diagnostiikan lisäksi on tärkeää löytää ennusteellisia tekijöitä, jotta uusia hoitomenetelmiä voidaan kehittää ja näin parantaa potilaiden ennustetta sekä estää elämänlaadun heikentyminen. HPV:n kiistaton merkitys suunielusyövässä on lisännyt kiinnostusta muihin onkogeenisiin viruksiin, kuten Epstein-Barr virukseen (EBV) ja polyomaviruksiin. Tieto niiden vaikutuksesta suunielusyövässä on kuitenkin vähäistä ja ennusteellinen rooli on tuntematon. Muut kliiniset ja biomolekulaariset tekijät, kuten syöpäkudostilavuus sekä matriksin metalloproteinaasit (MMP) ja niiden kudossäätelijät (TIMP) ovat potentiaalisia ennusteellisia merkkiaineita pään ja kaulan syövissä, mutta niiden rooli HPV-positiivisessa ja HPV-negatiivisessa suunielusyövässä on epäselvä.

Tutkimuksen tavoitteet

Tämän väitöskirjan ensimmäinen osa keskittyi diagnostiikkaan (osatyöt I-III). Tavoitteenamme oli parantaa HPV-määrityksen tarkkuutta tutkimalla eri HPV-määritysmenetelmiä suhteessa standardimenetelmään eli p16-proteiinin immunohistokemialliseen värjäykseen (osatyö I). Lisäksi halusimme ymmärtää paremmin taudin kliinistä ilmentymistä ja eri virusten roolia suunielusyövässä (osatyöt II-III). Määritimme HPV-positiivisten ja HPV-negatiivisten potilaiden ensioireet ja kliiniset piirteet sekä tutkimme eri virusten esiintymistä suunielusyövässä mukaan lukien EBV ja polyomavirukset.

Väitöskirjan toinen osa keskittyi mahdollisiin ennusteellisiin tekijöihin (osatyöt III-V). Halusimme tutkia, onko EBV:llä ja eri polyomaviruksilla ennusteellista vaikutusta suunielusyövässä (osatyö III). Lisäksi halusimme selvittää, onko primaarikasvain- ja kaulametastaasitilavuuksilla ennusteellista vaikutusta HPV-positiivisessa ja HPV-negatiivisessa suunielusyövässä. Tarkoituksenamme oli myös selvittää MMP-8 ja TIMP-1 molekyylien ennusteellista vaikutusta.

Aineisto ja menetelmät

Kaikkien osatöiden potilasmateriaali koostui uusista suunielusyöpäpotilaista, jotka oli diagnosoitu Helsingin yliopistollisessa sairaalassa vuosina 2012-2016. Lisäksi osatyö I sisälsi myös potilaskohortin vuosilta 2000-2009. Kliiniset tiedot kerättiin potilastietojärjestelmästä. HPV määritettiin syöpäkudosnäytteistä ja riippuen tutkimuksesta eri HPV-menetelmät vaihtelivat. Eri HPV-määritysmenetelmiä olivat p16-proteiinin immunohistokemiallinen värjäys, HPV deoksiribonukeliinihapon (DNA) määritys polymeraasiketjureaktiolla (PCR) ja lisäksi HPV DNA-/mRNA -määritykset tehtiin in situ hybridisaatiolla (ISH). EBV ja polyomavirukset määritettiin PCR:llä osatyössä III. Lisäksi tutkittiin onkogeenisen EBV:n eli EBER:in esiintymistä. Osatyössä IV tuumoritilavuudet laskettiin ennen hoitoja ja kaikki potilaat hoidettiin (kemo)sädehoidolla. TIMP-1 ja MMP-8 määritykset tehtiin sekä syöpäkudosnäytteistä että prospektiivisesti kerätyistä verinäytteistä.

Tulokset

HPV mRNA ISH osoittautui tarkimmaksi menetelmäksi aktiivisen HPV:n olemassaolon määrittämiseksi syöpäkudosnäytteissä (osatyö I). Suurimmalla osalla HPV-positiivisista potilaista ensioire oli kaulapatti, kun taas kipu oli yleisin ensioire HPV-negatiivisilla suunielusyöpäpotilailla (osatyö II). Vastaavasti muissakin kliinispatologisissa tekijöissä oli huomattavia eroja näiden kahden syöpätyypin välillä ja yli puolet potilaista oli HPV-positiivisia ollen selkeä valtavirus (osatyöt II-III). Polyomaviruksia löytyi syöpäkudosnäytteistä, mutta niillä ei ollut ennusteellista merkitystä. Sen sijaan EBER sijaitsi lymfosyyteissä lähellä syöpäkudosta ja korreloi merkittävästi HPV-positiivisuuden kanssa (osatyö III). EBER-positiivisilla mutta HPV-negatiivisilla suunielusyöpäpotilailla oli merkittävästi huonompi ennuste verrattuna HPV-positiivisiin potilaisiin (osatyö III). Suuri kaulametastaasitilavuus oli huonon ennusteen merkki sekä p16-positiivisilla että p16-negatiivisilla potilailla (osatyö IV). Lisäksi p16-negatiivislla suuri primaarikasvaintilavuus oli yhteydessä huonoon ennusteeseen. Tilavuuksien antama tarkkuus oli parempi verrattuna uusimpaan TNM-luokitukseen. Seerumin korkea TIMP-1 pitoisuus korreloitui huonoon ennusteeseen HPV-

negatiivisilla potilailla (osatyö V). MMP-8:llä ei ollut ennusteellista vaikutusta.

Päätelmät

Tuloksiemme mukaan mRNA ISH -menetelmä osoittautui erinomaiseksi ja muita menetelmiä paremmaksi määrittämään aktiivisen HPV:n olemassaoloa suunielusyöpäkudosnäytteistä. p16:sta tarkkuus HPV-määritysmenetelmänä on todettu alentuneeksi, sillä se voi olla positiivinen ilmankin HPV:ta. Tämän vuoksi on suositeltavaa varmistaa HPV:n olemassaolo p16-positiivisista näytteistä vielä mRNA ISH -tekniikalla, jolloin diagnostinen tarkkuus paranee ja suunielusyöpäpotilaat todennäköisemmin erotellaan tarkemmin HPV-positiivisiin ja HPV-negatiivisiin. HPV osoittautui selkeäksi valtavirukseksi ja ainoaksi, joka selkeästi jakaa suunielusyöpäpotilaat kahteen eri ryhmään niin kliinispatologisten tekijöiden kuin ennusteenkin suhteen. Onkogeenisellä EBV:llä, tarkemmin EBER:llä voi kuitenkin olla oma osuus suunielusyövässä, sillä sen positiivisuus korreloi merkittävästi HPV:n kanssa ja oli huonon ennusteen merkki HPV-negatiivisilla potilailla. Kasvaintilavuus ja seerumin TIMP-1 pitoisuus voivat mahdollisesti toimia itsenäisinä ennusteellisina tekijöinä etenkin HPV-negatiivisilla, joilla tiedetään edelleen olevan huono ennuste.

5. INTRODUCTION

Head and neck (HN) cancer is the 7th most common cancer worldwide and the majority of all subtypes are associated with male gender (1). The main two risk factors for HN squamous cell carcinoma (SCC) development are tobacco smoking and heavy alcohol use (2, 3). In recent decades the incidence of oropharyngeal squamous cell carcinoma (OPSCC) has been rapidly increasing, particularly in Western countries (4-7) despite reductions in tobacco smoking (8, 9). The main reason behind this phenomenon is high-risk (HR) human papillomavirus (HPV) (10-12). HR-HPV-positive OPSCC differs in its genetic and biological profiles and in its clinicopathological behavior from its HPV-negative counterparts (12-21). In addition, patients with HPV-positive OPSCC have a significantly more favorable prognosis than patients with HPV-negative OPSCC (7, 22-24). Currently, more than half of newly diagnosed OPSCCs in Northern Europe and USA are related to HPV (6, 7, 10, 25, 26).

The oropharynx can be divided into the following anatomic sites: tonsils, base of tongue, soft palate, and posterior pharyngeal wall (27). Tumors (including SCC) in these sites can grow imperceptibly and both HPV-positive and HPV-negative OPSCC are usually diagnosed at an advanced stage, thus typically requiring multimodality treatment (22, 23, 28-30). Aggressive treatment approaches, such as chemoradiotherapy (CRT), often lead to high iatrogenic side effects and impaired quality of life (31, 32). Hence, an improved understanding of presenting symptoms and signs of patients with HPV-positive and HPV-negative OPSCC should improve patient outcomes and quality of life.

The most commonly used method to detect HPV in tumor samples is immunohistochemistry (IHC) of $p16^{INK4A}$ protein. As a surrogate marker, $p16^{INK4A}$ (p16) overexpression is interpreted as a sign of HPV infection (33-36). The most recent 8th edition of the Union of International Cancer Control (UICC)/American Joint Committee on Cancer (AJCC) tumor-node-metastasis (TNM) classification distinguishes OPSCC into HPV-positive and HPV-negative disease entities by p16 status. This is because p16 status is easily available and highly sensitive in detecting HPV (37-39). However, even though p16 overexpression is highly sensitive it is less specific for HPV detection (33, 40). Although a wide range of other HPV-detection methods exists with variable sensitivity and specificity levels (40-42) there is no current consensus on the preferred methodology. Accurate methodology is essential not only for diagnostics but also to guide new therapeutic approaches for both HPV-positive and HPV-negative OPSCC patients to achieve more favorable outcomes (43).

In addition, new clinical and biomolecular prognostic markers are also warranted to improve patient outcomes. Primary gross tumor volume (pGTV) and nodal gross tumor volume (nGTV) have shown potential to serve as clinical prognostic factors in different HNSCCs (44-47). The benefits are due to a precise three-dimensional (3D) structure of the tumor, which thus allows more accurate tumor definition (44, 45, 48). However, comparative studies between patients with HPV-positive and HPV-negative OPSCC do not exist.

Regarding biomolecular prognostic markers, matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are enzymes that have an essential role in tumor pathogenesis and immune surveillance and are also associated with patient survival in different cancers (49-52). The prognostic role of MMPs and TIMPs have been partly evaluated in different HNSCCs. However, the prognostic role of these markers in HPV-positive and HPV-negative OPSCC remains unknown.

As HPV has an indisputable role and is a significant prognostic factor in OPSCC, interest in other oncoviruses in HNSCC has been increasing. Epstein-Barr virus (EBV) has shown a remarkable role in nasopharyngeal carcinoma (NPC) and other oncoviruses (such as polyomaviruses) have also been detected in HNSCC (53-56). However, little is known about other oncoviruses in OPSCC and knowledge on their prognostic role is scarce.

The aim of this thesis was to compare different HPV-detection methods against p16 IHC and to investigate if an additional method combined with p16 would improve diagnostic accuracy. In addition, we wanted to evaluate the presenting symptoms and clinical behavior of HPV-positive and HPV-negative patients and the presence and prognostic role of other oncoviruses in OPSCC. Further, we wanted to study the potential prognostic role of tumor volume and MMP-8 and TIMP-1 in both HPV-positive and HPV-negative OPSCC patients.

6. REVIEW OF THE LITERATURE

6.1 Epidemiology and etiology of oropharyngeal squamous cell carcinoma

Globally, over 850000 new cancer cases and 450000 deaths are attributed to head and neck (HN) cancer annually (1). Over 90% are histologically SCC (2) and heavy alcohol use and tobacco smoking are the main risk factors for developing a HNSCC (2, 3). Currently, oropharyngeal cancer (OP) accounts for approximately 90000 new cases annually worldwide and the majority is diagnosed in males (1). In recent decades the incidence of oropharyngeal squamous cell carcinoma (OPSCC) has been rapidly increasing, especially in many Western countries including Finland (4-7) despite reductions in tobacco smoking (8, 9). In Finland, the most common type of HNSCC is still oral cancer but the incidence of OPSCC has been increasing constantly (57). Currently, close to 200 new OPSCCs are diagnosed annually, whereas in the 1990s approximately 50 new cases were diagnosed annually (Figure 1) (57). The main reason behind this phenomenon is HR-type HPV, predominantly HPV-type 16 (5, 10, 11, 22, 58). More than half of newly diagnosed OPSCCs are HPV-related in the USA and in many other Western countries (6, 10, 25, 26).

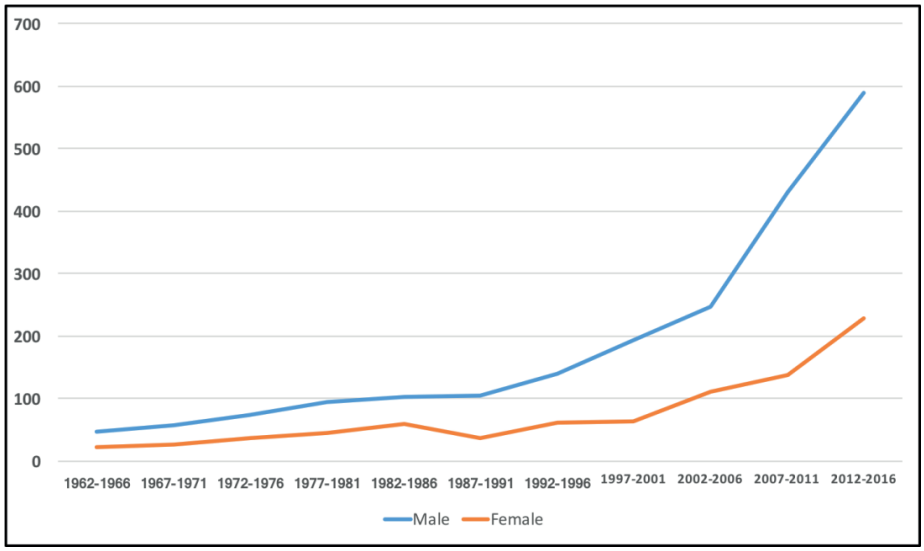

Figure 1. *New oropharyngeal cancer cases in Finland at 5-year intervals between 1962 and 2016 (57).*

HPV is a small, double-stranded DNA virus that comprises over 200 low-risk (LR) and HR genotypes (59, 60). The oncogenic HR-HPV-genotypes are as follows: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, and 70 (59). Oncogenic HR-HPVs are the predominant cause of cervical cancer (59) and are related to other malignancies, including OPSCC (22, 61). In OPSCC, the oncogenic HR-HPVs can independently lead to genetic instability and carcinogenesis without the established risk factors of heavy alcohol use and tobacco smoking, which are strongly related to the development of HPV-negative OPSCC (11). In general, HPV has been regarded as a sexually transmitted virus and a high number of sex partners is associated with HPV-positive OPSCC (11). However, it is notable that T-cells characteristic for HPV16 have also been observed in small children below sexually active age due to possible previous exposure to HPV16 infection (62).

Patients with HPV-positive OPSCC differ not only in etiologic backgrounds but also in demographics features when compared with their HPV-negative counterparts. Patients with HPV-positive OPSCC are more often male, typically of higher socioeconomic status, younger in age and are less likely to be heavy alcohol users or tobacco smokers when compared with HPV-negative patients (11, 22, 63-65). However, a substantial increase in the incidence of HPV-associated OPSCC among patients aged 70 and older has been recently reported (30), showing changing trends in the era of this HPV-driven disease. The incidence of HPV-positive OPSCC in the USA has been estimated to overtake the incidence of cervical cancer in 2020 (5).

6.2 Tumor site, histopathology, and pathogenesis

The oropharynx is distributed into four different anatomic subsites as follows: 1) tonsils (including tonsillar fossa, pillars, and lateral pharyngeal walls), 2) base of tongue, 3) soft palate, and 4) posterior pharyngeal wall (27). OPSCC can develop in each of these subsites (20). The tonsils and base of tongue contain lymphatic tissues and the majority of the HPV-positive OPSCCs are related to these subsites (21). The tonsils consist of a surface layer and an epithelial layer with a cryptic structure (12). The epithelial layer can bind to different antigens, such as HPV antigens. HPV antigens typically pass through into the basal cells of the tonsils via the crypts and the invasion of HR-HPV types may result in a malignant process. In HPV-negative OPSCC, the malignant invasive process begins in the normal epithelial structure of the surface layer in tonsils and other subsites of the oropharynx (12).

6.2.1 Histopathology

HPV-positive and HPV-negative OPSCCs have distinct histologic and morphologic features. Typically, HPV-positive OPSCCs are morphologically nonkeratinizing and consist of large cell nests with frequent mitoses and a scanty cytoplasm (66, 67). Instead, HPV-negative OPSCCs generally have keratinizing morphology (68). Tumors are classified by the level of differentiation into three different grades as follows: well, moderately, and poorly differentiated. Poorly differentiated represents the most aggressive histologic type and the majority of HPV-positive OPSCCs belong to this subgroup (68-70). Paradoxically, however, patients with HPV-positive OPSCC have a favorable outcome (22-24, 30). The reason for the nonkeratinizing morphology of HPV-positive tumors is not completely elucidated. It has been suggested that HPV-positive OPSCC displays a similar morphology as normal tonsillar crypts with immature structure of reticular epithelium; non-keratinizing SCC is therefore more likely a sign of a tumor derived from the tonsillar crypt (71, 72). This is consistent with the fact that HPV-positive tumors are not always nonkeratinizing and can arise from other sites of the oropharynx (20, 68).

6.2.2 Pathogenesis and intracellular pathways

Alterations in the function of various proteins, most importantly in tumor protein 53 (p53) and p16, have essential roles in the molecular carcinogenesis and intracellular pathways in OPSCC (15, 17, 18, 73). Tumor suppressor p53 is a protein that has a key role in the intracellular cell cycle and DNA repair and in inducing apoptosis (74). p16 is a tumor-suppressor protein encoded by the cyclin-dependent kinase inhibitor A2 (CDKN2A) gene and has an essential function in the cell cycle by especially inhibiting cyclin D1 (cD1) and CDK 4/6; p16 thus inhibits cell cycle progression from G1 to phase S1 (75). The mutation profiles and intracellular pathways, including the roles of p53 and p16, are different in HPV-positive and HPV-negative OPSCC (13, 15, 41).

6.2.2.1 HPV-positive OPSCC

HR-HPV has two viral oncoproteins, E6 and E7, which have distinct intracellular functions and ability to induce malignant cell conversion (17). These oncoproteins can disturb normal epithelial differentiation and cell cycle (such as apoptosis and DNA repair) by interacting with different host cell proteins (76). The main role of oncoprotein E6 is to bind with p53 protein, which leads to p53 degradation and loss of activity (17, 77). The oncoprotein E7 disturbs the cell cycle by binding with retinoblastoma protein (pRb) in a

phosphorylation-independent pathway, which in turn regulates function of the transcription factor E2F. The interaction between E7 and pRb leads to loss of pRb function, and thus amplification of E2F and changes in transcription. Upregulation i.e. overexpression of p16 protein attempts to inhibit this cascade by inhibiting phosphorylation of pRb but do not reach it due to the phosphorylation-independent pathway induced by oncoprotein E7 (18, 41). Consequently, p16 overexpression is characteristic for HR-HPV-positive malignancies (18). These intracellular changes due to oncoproteins E6 and E7 block DNA repair and apoptosis in cell cycle and lead to progression of malignant cells (17, 18). The intracellular pathway of HPV-positive OPSCC is presented in Figure 2. In addition, a recent study by Lawrence et al. (13) revealed that mutations in oncogene phosphoinositide-3-kinase, catalytic, alphapolypeptide (PIK3CA) and loss of TNF Receptor Associated Factor 3 (TRAF3) function that lead to activation of nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-kB) are related to HPV-positive OPSCC. This observation suggests that HPV-positive tumors may have several oncogenic pathways.

6.2.2.2 HPV-negative OPSCC

While the genetic alterations behind the pathogenesis of HPV-negative OPSCC are mainly attributed to tobacco smoking and heavy alcohol use, other risk factors such as poor oral hygiene and betel chewing have also been recognized (22, 73, 78-80). The intracellular effects regarding to these risk factors may lead to alterations in several genes, mainly in tumor suppressor genes tumor protein 53 (TP53), CDKN2A, and Cell Cycle Regulators Cyclin 1 (CCND1), which encode proteins p53, p16, and cD1, respectively (13, 15, 19). In addition, mutations in caspase 8, apoptosis-related cysteine peptidase (CASP8) and Harvey rat viral oncogene homolog (HRAS) and amplification of fas associated via death domain (FADD), yes-associated protein 1 (YAP1), and baculoviral IAP repeat containing 2 (BIRC2) genes have been recognized (13). In HPV-negative OPSCC, the mutation in gene TP53 leads to inactivation of p53, which imbalances normal cell cycle function such as DNA repair and apoptosis. The loss of p16 protein is related to HPV-negative tumors as p16 function is down-regulated by the deletion in CDKN2A gene. Amplification of gene CCND1 increases activity of cyclin-depedent kinases (CDKs) via elevated transcription of cD1, resulting in phosphorylation of pRb and E2F activation. This cascade induces instability in the cell cycle and leads to uncontrolled cell proliferation and replication in HPV-negative tumors (15). The intracellular pathway of HPV-positive OPSCC is presented in Figure 2.

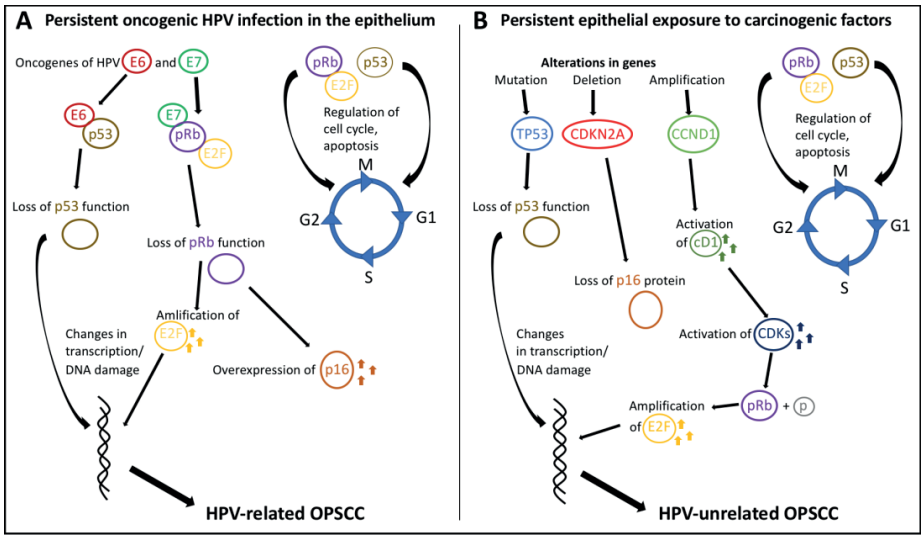

Figure 2. *Pathogenesis and intracellular pathways of HPV-positive (A) and HPV-negative (B) oropharyngeal squamous cell carcinoma.*
A: *Oncogenic HPV-protein E6 binds with p53, leading to degradation of p53. Oncogenic HPV-protein E7 binds pRB in a phosphorylation-independent pathway. Hence, pRB losses its function, leading to amplification of E2F. This cascade interrupts normal cell cycle and triggers DNA damage. E7-related loss of pRb function leads to p16 overexpression.*
B: *Alterations in genes TP53, CDKN2A and CCND1 leads to carcinogenic cascade, causing HPV-negative OPSCC. Mutation in TP53 leads to loss of p53 function and changes in normal cell cycle and transcription. Function of p16 is down-regulated via deletion in CDKN2A. Amplification of CCND1 leads to activation of cD1, resulting in phosphorylation of pRB and amplification of E2F. Amplification of E2F leads to DNA damage and changes in transcription.*

6.3 Diagnosis and diagnostic methods

6.3.1 Clinical presence

The clinical presentation and behavior are distinct between HPV-positive and HPV-negative OPSCC. HPV-negative tumors are more likely to extend locally in the primary tumor site in all oropharyngeal subsites, whereas HPV-positive primary tumors are often smaller, have a tendency to arise from tonsils, and spread early to cervical lymph nodes (20, 23, 81). In general, OPSCC is often diagnosed at an advanced stage and thus requires aggressive treatment approaches and may lead to impaired survival (22, 23, 30). Fortunately, despite the frequent nodal involvement at the time of diagnosis, patients with

HPV-positive OPSCC have a significantly more favorable prognosis compared with their HPV-negative counterparts (22, 23). However, delay in diagnosis is a moderate risk factor for increased mortality in OPSCC and several reasons for diagnostic delay have been suggested (20, 28, 82). One theory is that tumors <2 cm in diameter do not often present local symptoms due to its small size (28). Recent studies support this observation in both HPV-positive and HPV-negative OPSCC (20, 29). According to these studies, the majority of patients with HPV-positive tumors had a neck mass as the initial symptom and most of the primary tumors were <2 cm in diameter. Instead, the most common initial symptom among patients with HPV-negative tumors was sore throat and most tumors were >2 cm in diameter at the time of OPSCC diagnosis (20, 29). Another important reason for delayed diagnosis is that patients often assume that initial symptoms such as sore throat, odynophagia, otalgia, or a neck mass are related to benign processes and will pass with time (20, 83). Furthermore, healthcare professionals often incorrectly assume that a neck mass in HPV-positive OPSCC is part of a benign process such as cyst or infection, thus resulting in delayed diagnosis (20, 29, 84, 85). An improved knowledge in presenting symptoms and signs is necessary to improve diagnostics and decrease diagnostic delays of both HPV-positive and HPV-negative OPSCC.

6.3.2 Tumor evaluation and diagnostics

In clinical practice, biopsies are taken from suspicious oropharyngeal areas during an otorhinolaryngological and endoscopic examination or under general anesthesia. Magnetic resonance imaging (MRI) or computer tomography (CT) of the HN area is performed routinely for every patient. MRI has advantages in soft tissue extension evaluation whereas CT is precise in bony invasion assessment (86). OPSCC diagnosis is assessed according to the typical histopathological findings in tumor samples, followed by routine HPV testing (most commonly by p16 IHC) (66, 87). In case a neck mass is the only clinical finding (i.e. unknown primary), ultrasound and fine-needle aspiration cytology are taken from the cervical lymph node to reveal possible signs of SCC (88). Unknown primaries are tested for HPV by p16 IHC and a positive result is a strong sign of oropharyngeal origin of the primary tumor (89, 90). To search for OPSCC diagnosis, a HPV-positive lymph node finding is followed by bilateral tonsillectomy and random biopsies are taken from oropharynx subsites (88). Potential distant metastases are usually evaluated by CT.

6.3.3 HPV detection

HPV can be detected from tumor tissue samples by various different methods, including p16 IHC, DNA or RNA ISH, DNA polymerase chain reaction (PCR), or by detection of HPV E6/E7 mRNA transcripts by quantitative reverse transcriptase-PCR (qRT-PCR) (33, 40, 41, 91, 92). The main purpose of all methods is to verify a biologically active HR-HPV in tumor tissue samples. Each of these techniques has its own strengths and limitations, such as differences in laboratory expenses and detection accuracy between different pathologists and variable sensitivity and specificity levels (41, 42, 92). None of the methods individually has shown superiority in clinical practice as a diagnostic tool for HPV detection. However, combining different methods has improved the sensitivity and specificity of HPV detection (33, 40) but no combinations or protocols have become part of routine diagnostics. HPV-positive OPSCC patients have a significantly more favorable prognosis than their HPV-negative counterparts, which has been established in numerous studies (22-24, 30, 93, 94). Therefore, there are several ongoing trials considering treatment de-escalation for HPV-positive patients (43, 95). Verified and reliable HPV methodology is essential, particularly for such de-escalation strategies. The different HPV methods are described in more detail in the next sections and their advantages and disadvantages are presented in Table 1.

6.3.3.1 p16 immunohistochemistry

The most applied method for detecting HPV in OPSCC from paraffin-embedded formalin-fixed (FFPE) tumor samples is immunostaining of p16 protein (33-36), as p16 activation in intracellular pathways is characteristic for HPV-positive OPSCC (18, 96). p16 is a surrogate marker as its overexpression correlates to biologically active oncogenic HPV infection (34, 35). According to WHO recommendations, the tumor sample is considered p16-positive if >70% of the cells are diffusively positive by IHC (97). p16 IHC is easy to perform, cost-effective, highly sensitive, and a widely available method for detecting HPV in tumor samples (36). Therefore, p16 IHC is stated as the HPV detection method to stratify HPV-positive tumors from HPV-negative as described in the recent update of AJCC 8th edition of TNM classification (37, 38). However, although p16 IHC is highly sensitive it is less specific for HPV detection in OPSCC and therefore has been criticized as a stand-alone diagnostic test (33, 40). It has been suggested that pRb can be inactivated and p16 overexpressed not only by an HPV-induced pathway but due to other mechanisms and oncogenic pathways, such as point mutations, gene deletions, and even other viruses (43, 75). Another argument for using p16 IHC only to detect HPV is that OPSCC patients with p16-positive but HPV DNA PCR-negative tumors is due to the to poorer prognosis compared with OPSCC

patients with double-positive tumors (p16+/HPV DNA+) (36, 94). Furthermore, although p16 is sensitive for HPV detection in OPSCC, it lacks sensitivity in other HNSCC (98).

6.3.3.2 Detection of HPV DNA by PCR

The widely used HPV DNA detection method is based on determining amplification of viral DNA by PCR. PCR is a highly sensitive HPV-detection method and can be performed from FFPE tumor tissue samples (33, 40). Depending on the study kit, a wide range of different HPV LR and HR genotypes can be detected (99-101). However, detection of viral DNA by PCR is not sufficiently specific as it does not distinguish between transcriptionally active and clinically irrelevant HPV infections (92, 102). Another limitation of PCR as a laboratory technique is the risk of contamination (103, 104). Hence, PCR is not recommended to be used as a single test in routine diagnostics. However, when PCR is combined with p16 IHC, the HPV detection specificity improves while sensitivity remains sufficient (33, 40).

6.3.3.3 Detection of HPV mRNA by qRT-PCR

Detection of HPV E6/E7 mRNA transcripts by qRT-PCR indicates transcriptionally active HPV in fresh frozen or FFPE tumor tissue samples (105, 106). It is highly specific and is considered the gold standard method for HPV detection (87, 105, 106). However, the method is technically laborious as it usually requires microdissection of fresh frozen samples (33, 98). In addition, routine OPSCC diagnostics are frequently performed from FFPE tumor tissue samples and not from fresh frozen samples (33, 98). In FFPE samples, RNA is often fragmented and damaged and thus requires laborious and expensive techniques to recover and prepare RNA for qRT-PCR (33, 92, 102). Consequently, detection of HPV E6/E7 mRNA by qRT-PCR is not part of a routine diagnostic protocol.

6.3.3.4 Detection of HPV DNA by ISH

HPV DNA detection by ISH permits direct visualization of tumor cell nuclei in FFPE tumor tissue samples and is thus a highly specific method (40, 107, 108). However, this method has insufficient sensitivity as it does not always recognize HPV when the viral load is low (40, 107, 108). Therefore, HPV DNA detection by ISH cannot be performed as a single test for HPV evaluation in clinical practice.

6.3.3.5 Detection of HPV mRNA by ISH

HR-HPV E6/E7 mRNA transcripts can also be identified directly from FFPE tumor tissue samples by ISH-based methods (i.e. RNAscope HPV-test) (98, 102). The test is highly specific for detecting HR types of HPV as it assesses only clinically relevant genes (i.e. E6/E7) (102). Furthermore, this method is technically feasible and can thus be performed in routine laboratory diagnostics (98). Therefore, increasing evidence exists on its ability to serve as a diagnostic tool in clinical practice (92, 107).

Table 1. HPV-detection methods (33, 34, 40, 42, 98, 102)

Method	**Methodology**	**Advantages**	**Disadvantages**
p16 IHC	Based on the overexpression of p16 protein by IHC	Sensitivity, highly available	Specificity
DNA PCR	Amplification of viral DNA determined by PCR	Sensitivity	Specificity, risk for contamination
mRNA qRT-PCR	HPV E6/E7 mRNA transcripts detected by qRT-PCR	Specificity, sensitivity, detects transcriptionally active HPV	Technically laborious, typically performed from frozen samples
DNA ISH	DNA detected in tumor cell nuclei by ISH	Specificity	Sensitivity
mRNA ISH	Based on direct visualization of genes E6/E7	Sensitivity, sensitivity, detects transcriptionally active HPV	Lack of comparative studies and experience

Abbreviations: IHC = immunohistochemistry, ISH = in situ hybridization, qRT-PCR = quantitative reverse transcriptase-polymerase chain reaction.

6.4 Staging and treatment

6.4.1 TNM classification and staging

In OPSCC, tumors were previously classified and staged according to the 7th edition of AJCC/UICC TNM classification by primary tumor size and possible invasion to nearby structures, regional nodal involvement, and distant metastases (Table 2) (109, 110). However, the 7th edition of AJCC TNM classification did not take into account the presence and behavior of HR-HPV-

related OPSCC. HR-HPV-positive OPSCC differs remarkably in clinicopathological behavior and prognosis from HPV-negative OPSCC; these are thus two different disease entities. Therefore, an update to TNM classification was warranted. In 2016, the newest 8th edition of AJCC/UICC TNM staging was launched and presented different classifications for HPV-positive and HPV-negative OPSCC based on p16 status (Table 3 and 4) (37-39). For p16-positive OPSCC, T and N classifications were updated and the staging changed markedly towards clinically less advanced disease. For p16-negative tumors, N-classification and staging were updated and now include extranodal extension.

Table 2. 7th edition of UICC and AJCC TNM staging of oropharyngeal squamous cell carcinoma (109, 110)

T category	**T description**
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Ti	Carcinoma *in situ*
T1	Tumor <2 cm in greatest dimension
T2	Tumor >2 cm but ≤4 cm in the greatest dimension
T3	Tumor size ≥4 cm in the greatest dimension or extension to lingual surface of epiglottis
T4	Moderately/very advanced disease
T4a	Tumor invades the larynx, deep or extrinsic muscle of the tongue, medial pterygoid, mandible, or hard palate
T4b	Tumor invades the lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, or skull base or encases the carotid artery

N category	**N description**
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node <3 cm in the greatest dimension
N2 (a-c)	Metastases >3 cm but ≤6 cm in the greatest dimension in ipsilateral, contralateral, or bilateral lymph nodes
N2a	Metastasis in a single ipsilateral lymph node >3 cm but ≤6 cm in the greatest dimension
N2b	Metastasis in multiple ipsilateral lymph nodes ≤6 cm in the greatest dimension
N2c	Metastasis in bilateral or contralateral lymph nodes ≤6 cm in the greatest dimension
N3	Metastasis in lymph node >6 cm in the greatest dimension

M category	M description
MX	Distant metastases cannot be assessed
M0	No distant metastases
M1	Distant metastases

Stage			
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T1	N1	M0
	T2	N1	M0
	T3	N0/N1	M0
Stage IVA	T1-T3	N2	M0
	T4a	N0-N2	M0
Stage IVB	Any T	N3	M0
	T4b	Any N	M0
Stage IVC	Any T	Any N	M1

Clinical and pathologic TNM classification and staging are the same. M1 disease is Stage IV.

Table 3. 8th edition of UICC and AJCC TNM staging of p16-positive oropharyngeal squamous cell carcinoma (38, 39)

T category (Clinical/Pathologic)	**T description**
T0	No evidence of primary tumor
T1	Tumor <2 cm in greatest dimension
T2	Tumor > 2 cm but ≤4 cm in the greatest dimension
T3	Tumor size ≥4 cm in the greatest dimension or extension to lingual surface of epiglottis
T4	Tumor invades the larynx, deep or extrinsic muscle of the tongue, medial pterygoid, hard palate, or mandible or beyond

N category (Clinical)	**N description**
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	One or more ipsilateral lymph nodes, each size ≤6 cm
N2	Contralateral or bilateral lymph nodes, each size ≤6 cm
N3	Lymph node(s) ≥6 cm

N category (Pathologic)	**N description**
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastases in ≤4 lymph nodes
N2	Metastases in >4 lymph nodes

Stage (Clinical)					**Stage (Pathologic)**			
	N0	N1	N2	N3		N0	N1	N2
T0	NA	I	II	III	T0	NA	I	II
T1	I	I	II	III	T1	I	I	II
T2	I	I	II	III	T2	I	I	II
T3	II	II	II	III	T3	II	II	III
T4	III	III	III	III	T4	II	II	III

M1 disease is Stage IV.

Table 4. 8th edition of UICC and AJCC TNM staging of p16-negative oropharyngeal squamous cell carcinoma (38, 39)

T category	**T description**
TX	Primary tumor cannot be assessed
Tis	Carcinoma *in situ*
T1	Tumor <2 cm in greatest dimension
T2	Tumor >2 cm but ≤4 cm in the greatest dimension
T3	Tumor size ≥4 cm in the greatest dimension or extension to lingual surface of epiglottis
T4	Moderately/very advanced disease
T4a	Tumor invades the larynx, deep or extrinsic muscle of the tongue, medial pterygoid, mandible, or hard palate
T4b	Tumor invades the lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, or skull base or encases the carotid artery

N category	**N description**
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Single ipsilateral lymph node, ≤3 cm and ENE-negative
N2a	Single ipsilateral lymph node, >3 cm/≤6 cm and ENE-negative
N2b	Multiple ipsilateral lymph nodes, ≤6 cm and ENE-negative
N2c	Bilateral or contralateral lymph nodes, ≤6 cm and ENE-negative
N3a	Metastasis in a lymph node, >6 cm and ENE-negative
N3b	Any ENE-positive lymph node(s)

Stage				
	N0	N1	N2a,b,c	N3a,b
T1	I	III	IVA	IVB
T2	II	III	IVA	IVB
T3	III	III	IVA	IVB
T4a	IVA	IVA	IVA	IVB
T4b	IVB	IVB	IVB	IVB

ENE = extranodal extension. Clinical/pathologic T and N categories are the same as well as stage. M1 disease is Stage IVC.

6.4.2 Treatment

The chosen treatment approach for OPSCC depends on the primary tumor site, disease extension (TNM), p16 status, and patient-related factors such as comorbidities, compliance, and the expected impact of treatment on quality of life. The principal treatment modalities for OPSCC are as follows: 1) definitive radiotherapy (RT) with or without chemotherapy, 2) surgical resection of primary tumor including neck dissection (ND) when needed, and 3) surgical resection with adjuvant RT with or without chemotherapy (7).

6.4.2.1 Surgery

The primary tumor resection is typically performed with an open approach transorally (7). In addition, endoscopic transoral robotic surgery (TORS) is becoming more popular due to its advantages in challenging sites by providing a 3D visual structure of the tumor (111). Extensive open surgical approaches for primary tumors may be warranted even through the neck to achieve clear surgical margins (112). Radical surgery may lead to wide soft tissue defects resulting in large soft tissue reconstruction that typically impair swallowing and quality of life. Neck metastases are often present and can be managed either with ND or RT (113). The extension of ND is classified to different levels and depends on the primary tumor site and T class and N class (112, 114). In a case of clinically N0 disease (no sign of neck metastases), the neck is recommended to be managed electively by ND or RT if the risk of neck metastases is considered >20 % (115).

6.4.2.2 (Chemo)radiotherapy

RT can be used as a definitive treatment modality for OPSCC, an adjuvant therapy after primary surgery, or as a palliative therapy. RT is based on ionizing radiation that leads to DNA damage and thus loss of cell function. It is typically highly sensitive for tumor tissue but nevertheless has an undesirable impact on normal tissue. The HN area consists of essential organs, including the whole upper airway tract and crucial structures for swallowing. Therefore, RT for HN tumors should be as precise as possible to avoid damaging nearby structures. During the few last decades RT has improved significantly. Currently, HN tumors are pre-evaluated in 3D by CT scan for RT dose planning. The most common type of RT is intensity-modulated radiotherapy (IMRT). IMRT allows multiple radiation beam directions and accurate targeting of high radiation doses to the tumor with minimal impact on nearby structures. For OPSCC, IMRT is given in daily 2-gray (Gy) fractions

and the total dose is between 60 to 70 Gy depending on tumor- and treatment-related factors. Elective areas are treated with 50 Gy (116).

In OPSCC, IMRT is frequently intensified by chemotherapy (22, 23). Chemotherapy is typically cisplatin-based and administered at a 40 mg/m_2 weekly dose. In case cisplatin is contraindicated, chemotherapy may be performed with other medicines such as paclitaxel or carboplatin (116). Chemotherapy is typically performed concomitantly as it is more effective than neoadjuvant chemotherapy (117).

6.4.2.3 Follow-up and recurrent disease

Response to treatment is evaluated with a clinical examination and positron emission tomography (PET)-CT typically 3 to 4 months after conclusion of primary treatment. PET-CT is highly sensitive for screening for residual tumor or recurrent disease (118). Salvage surgery may be performed when possible for biopsy-confirmed residual tumor or recurrent disease (119, 120). In addition, palliative therapy including RT or surgery are options in case curative treatment is not feasible (121).

6.4.2.4 Future directions

The main goal for the development of treatment modalities in OPSCC is to achieve high treatment response with as low as possible iatrogenic toxic effects to ensure favorable long-term quality of life. The favorable prognosis and treatment response (particularly to CRT) among patients with HPV-positive OPSCC is well established (22, 23). However, treatment-related side effects such as mucositis, xerostomia, and chronic dysphagia are major iatrogenic issues that lead to reduced quality of life (31, 32). Therefore, there are several ongoing trials focusing on de-escalated surgical and CRT/RT treatment approaches for HPV-positive OPSCC (95, 122, 123).

Another interesting approach in the field of treatment modalities is immunotherapy. It has been suggested that immunotherapeutic targets between patients with HPV-positive and HPV-negative OPSCC are different (124). The oncoproteins E6 and E7 have been speculated to serve as the main immunotherapeutic targets and the immune checkpoint protein programmed death-1 (PD-1) that prevents T-cell function is related to HPV-positive OPSCC (124, 125). T cells via adaptation, NK cells, and dendritic cells have been suggested to serve as the main molecular targets for immune-based therapy in HPV-negative HNSCC (124).

Increasing evidence exists on the benefits of proton therapy (PT), particularly intensity-modulated PT as a treatment modality for both definitive and recurrent disease (126). PT is based on beam of protons irradiated directly to a precise depth, thus minimizing effects on nearby structures. Therefore, toxic side effects are significantly lower compared with IMRT. However, significant differences in patient survival have not been found between these two treatment modalities (126). PT has limitations in availability and cost but may achieve a stronger position in clinical practice after a potential increase in availability and due to favorable experience (126).

6.5 Prognosis and prognostic factors

6.5.1 HPV and prognosis

During the last few decades the survival of OPSCC patients has improved remarkably in many Western countries, including Finland (7, 23, 24, 127). The role of HR-HPV behind this trend is well established, as HPV-positive OPSCC patients have a significantly more favorable prognosis compared with their HPV-negative counterparts regardless of treatment modality (7, 22-24, 128). The better survival may be attributed to virus-related antitumor activity and immune control (129, 130). In general, patients with an HR-HPV-positive OPSCC have approximately 50% better disease-free survival (DFS) rates and a 30% lower risk of death when compared with HPV-negative OPSCC patients (24); a similar trend is also observed among elderly patients (30). Another important reason for increased survival rates is the development of treatment strategies, particularly in the case of IMRT which has shown improved patient outcomes (127, 131). In addition, concomitant chemotherapy with RT has significantly increased survival rates (117). Due to the excellent survival rates of HPV-positive OPSCC patients, treatment is evolving towards de-escalation (95, 122, 123). However, there is still a group of HR-HPV-positive OPSCC patients that do not respond to treatment. Smoking in particular has been attributed to impaired treatment response (22). In addition, the patient outcome varies on whether tumor HPV positivity was assessed solely according to p16 IHC positivity or to combined HPV testing by the different existing methods (43, 94).

Despite developments in treatment modalities, the survival rate of HPV-negative OPSCC patients has not improved significantly and remains unfavorable compared with HPV-positive OPSCC (22-24, 128). A large number of different biological and clinical factors has been investigated but none has shown as powerful an impact on prognosis as HPV. New clinical and molecular prognostic factors are necessary to personalize treatment and improve outcomes, particularly for HPV-negative OPSCC.

6.5.2 Other clinical and molecular prognostic factors

6.5.2.1 Tobacco smoking and heavy alcohol use

The association between tobacco exposure and development of HPV-negative OPSCC is well established (13, 15, 73, 78). Tobacco smoking induces multiple genetic transformations leading to carcinogenesis and thus to different malignancies, including OPSCC (13). Tobacco smoking independently impairs survival rates not only among HPV-negative but also among HPV-positive OPSCC patients (22, 132, 133). It has been speculated that tobacco exposure in HPV-positive OPSCC may alter it genetically more towards HPV-negative behavior and thus reduce sensitivity to CRT (22). Heavy alcohol use is not only a risk factor for OPSCC but is also related to an approximately 1.6- to 2.6-fold increase in mortality rates depending on the study (78, 134).

6.5.2.2 TNM classification and staging

According to the 7th edition of the TNM classification, stage, T-class, and N-class have shown independent prognostic value in OPSCC (135-138). However, the prognostic significance of the 7th edition TNM classification differs in HPV-positive and HPV-negative patients (139). Despite the favorable prognosis, HPV-positive OPSCCs are typically classified as higher stage (III-IV) according to the 7th edition of TNM classification (20, 29, 140). Due to differences in prognosis and behavior, an update for TNM classification and staging was warranted to distribute and stratify HPV-positive and HPV-negative OPSCC patients accordingly. The newest 8th edition of the TNM classification has better prognostic accuracy than the previous 7th edition (141, 142).

6.5.2.3 Tumor volume

pGTV and nGTV are promising prognostic markers in head and neck cancer (HNSCC), including OPSCC (44-48, 143-147). A higher pGTV may contain a higher number of clonogenic cells, leading to increased RT resistance which results in an elevated risk of recurrent disease and impaired survival rates (148-150). Therefore, it has been proposed that a higher dose of RT may be considered for patients with a larger tumor and metabolic volume to eliminate carcinogenic cells and to achieve better treatment response (147, 151).

Among HNSCC patients treated with definitive RT, tumor volume measurement has shown superiority a prognostic tool compared with the traditional TNM classification (7th ed.) (45, 47, 147, 152). TNM staging has

limitations in precise tumor evaluation as it is based on defining anatomic extension and the one-dimensional size of tumor (153, 154). Therefore, inaccurate measurement may categorize tumors with distinct volumes into the same stage, which may mislead diagnostics (44, 45, 147, 155). Definition of tumor volume provides a precise 3D structure of the tumor and hence improves evaluation of the total tumor mass (44, 45, 48).

pGTV and nGTV are reliable prognostic tools in evaluating locoregional control (LRC) and DFS after definitive IMRT in OPSCC (48, 155). However, all reports focused on tumor volume in OPSCC are based on the 7th edition of TNM classification and comparative studies of HPV-positive and HPV-negative OPSCC patients are not available. Tumor volume as a potential prognostic factor should be studied separately in HPV-positive and HPV-positive OPSCC patients due to significant differences in tumor behavior and prognosis between these two subgroups.

6.5.2.4 Matrix metalloproteinases and tissue inhibitors of metalloproteinases

MMPs are a group of proteolytic enzymes that have an have essential function in degradation of extracellular matrix (ECM) proteins (52). In general, degradation of ECM includes remodeling and tissue repair, but dysregulation of these processes may lead to various diseases such as cancer. It has been suggested that MMPs may have a direct cell-signaling impact on numerous cell-surface proteins (156, 157). A total of 24 different MMPs exist in humans and are regulated by TIMPs. Both MMPs and TIMPs are associated with the pathogenesis of various cancers (49, 50, 158, 159). MMPs and TIMPs regulate immune control, cell growth, and cell cycle, including apoptosis. Instability between MMPs and TIMPs may result in tumor invasion and extent of malignant disease (49, 51, 160, 161). Elevated expression of different MMPs has been recognized in various cancers by immunohistochemical analyses. This overexpression is typically associated with unfavorable prognosis (49, 162, 163). However, not all MMPs are related to cancer progression and impaired prognosis. For example, MMP-8 may have a tumor suppressing function by generating inflammatory mediators and its overexpression is associated with increased survival rates in different cancers (164-168). In contrast, elevated immunoexpression of TIMP-1, a specific inhibitor of MMP-8, is associated with poor prognosis in various types of cancer (169, 170). Consistent findings regarding TIMP-1 correlation with poor prognosis has been observed among patients with elevated TIMP-1 serum levels in different cancers, including HNSCC (51, 170-173). In addition to inhibiting MMP-8, TIMP-1 may also function as a growth factor by adhering to different cell-surface molecules, such as cluster of differentiation (CD)63, and thus disturb intracellular pathways (174, 175). This interaction may lead to activation of

intracellular focal adhesion kinase (FAK), which is associated with disturbed immune surveillance and increased cancer invasion (176, 177). The regulation of TIMP-1 on cytoplasmic membrane is illustrated in Figure 3. Both MMP-8 and TIMP-1 have been proposed as potential prognostic markers in different cancers, including HNSCC. However, their roles and impact on prognosis in HPV-positive and HPV-negative OPSCC have not been elucidated.

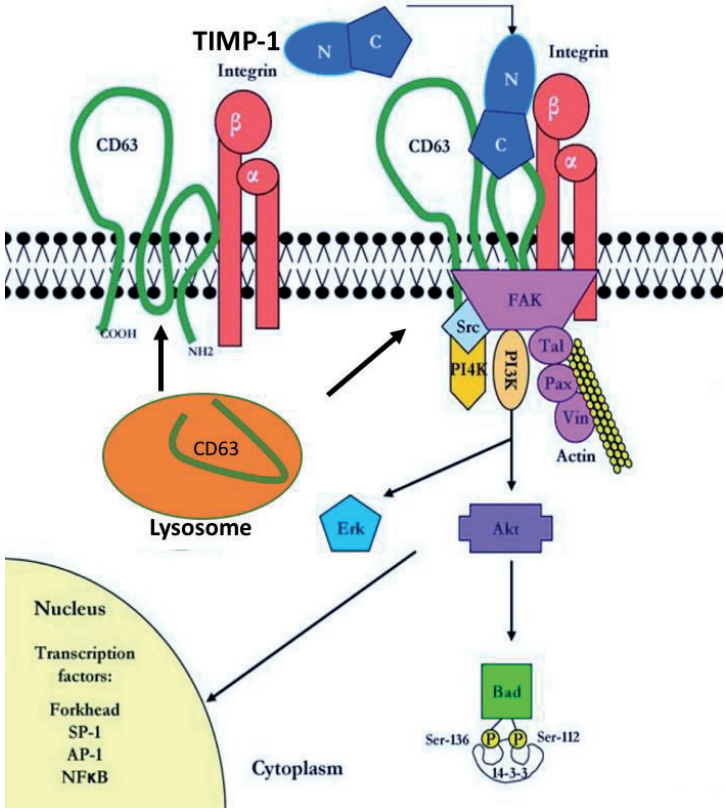

Figure 3. *The TIMP-1 regulation on cytoplasmic membrane and the impact of TIMP-1 on intracellular pathways. TIMP-1 binds CD63/integrin complex and the interaction activates integrin-mediated signaling pathway. This cascade activates focal adhesion kinase (FAK), Erk, PI 3-K and erk, leading to inhibiton of apoptosis and regulating transcription factors. Slightly modified and reprinted from the original publication (157) with permission from Springer.*

6.6 The role of other oncoviruses in OPSCC

Interest in various types of oncoviruses in HNSCC has increased due to the indisputable role of HPV in OPSCC. The presence and role of HPV in other HNSCC is not as remarkable as in OPSCC, although an association between HPV and improved prognosis in NPC has recently been shown (10, 178). Numerous other oncoviruses with distinct roles, such as Epstein-Barr virus (EBV) and different polyomaviruses, have been detected in HNSCC (22, 54, 56, 178-181). In addition, viral coinfections in HNSCC have been revealed in different reports. Such coinfections are of great interest as they may modulate tumor behavior to a more invasive process and may enhance resistance to treatment (56, 100, 182). A knowledge of different viruses in HNSCC is essential to better understand tumor behavior and to develop more personalized treatment strategies.

6.6.1 Epstein-Barr virus

EBV belongs to the group of herpes viruses that infects nearly all humans worldwide without leading to any significant health-related issues in the majority of cases (183). A latent EBV infection, specifically EBV-encoded small RNAs (EBERs), are related to various types of cancer (55, 184-186). The majority of NPCs are attributed to EBV and patients with EBV-attributed NPC have been associated witha better prognosis than those with EBV-negative tumors (178, 187-189). An oncogenic EBV infection, primarily via oncogenes latent membrane protein (LMP)-1, LMP-2A, and Epstein-Barr nuclear antigen 1 (EBNA1), is related to the pathogenesis and induction of NPC (190). The latter two oncogenes have been suggested to be potential targets for immunotherapeutic vaccines (55, 191). In addition, NPC due to EBV infection is a potential target for T-cell based immunotherapy (55, 192, 193). In contrast to NPC, patients with EBV-infected tumors have a poorer prognosis than those with EBV-uninfected tumors in other HNSCC, although the results were based on a relatively small patient cohort without focusing on a specific subsite (56). In OPSCC, EBV has been suggested to enhance invasiveness (182) and to work as a cofactor. However, EBV has only been studied in a few reports with a limited number of OPSCC patients and thus its role remains poorly understood (56, 182, 194).

6.6.2 Polyomaviruses

Similar to EBV, the presence of polyomaviruses is also rather common in humans and they are infrequently associated with disease (195). Polyomaviruses consist of early and late gene regions that encode T antigens and capsid proteins, respectively (195). Although polyomaviruses rarely cause disease, they have been detected in various cancers (including HNSCC) and the T antigens encoded by early gene regions are oncogenic (195). Detection of clonally integrated polyomavirus DNA in tumor tissue may signal involvement in a malignant process (195). The impact of Merkel cell polyomavirus on the carcinogenesis of Merkel cell carcinoma is irrefutable (54, 181, 196-199). Polyomaviruses are rarely direct drivers of pathogenesis in malignant diseases but frequently work as cofactors in cell transformation towards a malignant process. Accordingly, the oncogenic role of polyomaviruses is of a great interest (54, 200). The polyomaviruses John Cunningham virus (JCV) and BK virus (BKV) are proposed to be oncogenic and the latter is associated with urothelial malignancies (195, 201, 202). While DNA from both of these viruses have also been detected in HNSCC, their role in carcinogenesis and survival remains unclear (54). The presence of BKV and JCV in OPSCC is limited to only a few study cohorts with insufficient sample sizes and without evaluation of patient outcome (54, 99, 198). Another potential oncogenic polyomavirus is Simian virus 40 (SV40), which has been suggested to transform different human cells towards an oncogenic process (181). SV40 has been attributed to malignancies such as mesothelioma and Non-Hodgkin lymphoma (181, 200, 203). Studies in HNSCC, particularly in OPSCC, remain limited as thus far only one study has investigated SV40 presence in HNSCC in a limited number of patients (54).

6.7 Prevention of OPSCC

Targeted prevention is essential to stop the increase in incidence of HPV-related OPSCC and additionally to suppress induction of other cancers such as cervical, penile, and anal cancers attributable to HPV (204). Currently, three different prophylactic vaccines exist to prevent different HR-HPV types. HPV16 and HPV18 are covered by all three available prophylactic vaccines and HPV33 only by the 9-valent vaccine. The positive impact of these vaccinations in preventing the most predominant HPV-attributable cancer (i.e. cervical cancer) is well established in clinical trials (204-206). While efficacy in preventing HPV-associated HNSCC remains unproven by long-term population studies, it is estimated that up to 90% of all HPV-related cancers may be prevented by vaccination (204, 207). Promising results of potential HPV-vaccination efficacy in the oropharynx has recently been observed in the UK, as the national vaccination program is significantly associated with a

dramatic decrease in oropharyngeal HPV16 infections in children and young adults (208).

Worldwide, the main prevention strategies against cancer, including HPV-negative OPSCC, should be focused on reducing heavy alcohol use and tobacco smoking. In Finland, the Tobacco Smoking Act has shown a long-term decrease in smoking initiation in youth (8). Different interventions and policies against smoking and heavy alcohol use are warranted to reduce population-based exposure and to change attitudes towards these cancer risk factors (209).

7. AIMS OF THE STUDY

The first part of this study is focused on HPV methodology and the clinical presence of HPV and other oncoviruses in OPSCC. The second part of this study examined the prognostic role of oncoviruses in OPSCC and different clinical and biomolecular factors.

The specific objectives of this thesis were:

1. To compare different HPV methods against p16 IHC and to investigate if an additional method combined with p16 would improve diagnostic accuracy of HPV
2. To evaluate the presenting symptoms and signs of patients with HPV-positive and HPV-negative OPSCC
3. To investigate the presence and prognostic role of EBV and different polyomaviruses in OPSCC
4. To examine the prognostic role of pGTV and nGTV in HPV-positive and HPV-negative OPSCC
5. To observe the presence and the prognostic role of MMP-8 and TIMP-1 in tumor tissue and in prospectively collected serum samples both in HPV-positive and HPV-negative OPSCC

8. MATERIALS AND METHODS

8.1 Study I

Study I was based on two different consecutive OPSCC patient cohorts diagnosed and treated at the Helsinki University Hospital in two different time periods. Patients with previous HNSCC or a second primary cancer at the time of OPSCC diagnosis were excluded. The first patient material (PM I) was collected retrospectively and included 313 OPSCC patients diagnosed between January 1, 2000 and December 31, 2009, of which 202 patients had available tumor tissue samples for further laboratory analyses. Several studies on PM I have already been published. The second patient material (PM II) was collected prospectively and consisted of 224 OPSCC patients diagnosed between February 2, 2012 and March 3, 2016, of which 155 had available tumor tissue samples. A total of 357 OPSCC patients covering both materials were included in Study I.

HPV methodology was based on p16 IHC and HPV DNA ISH for PM I as described earlier. For PM II, the HPV methods were p16 IHC and HPV DNA PCR as partially discussed in Study II. For both materials, mRNA ISH was performed from tissue microarray (TMA) blocks. All methods were compared with p16 IHC results to assess the sensitivity and specificity levels of each method. Additionally, positive and negative predictive values were evaluated for each method.

Clinical and tumor-related factors were collected from hospital registries and compared with each HPV method. The data included the following parameters: age at time of OPSCC diagnosis, sex, smoking status, heavy alcohol use, and tumor grade. The TNM classification and staging were assessed according to the 7th edition and 8th edition of AJCC staging for PM I and PM II, respectively.

8.2 Study II

Study II consisted partly of the same patients as PM II in Study I. For Study II, 118 consecutive OPSCC patients diagnosed in a 2-year time period between February 2, 2012 and February 27, 2014 were included. HPV was assessed according to p16 IHC and HPV DNA PCR status.

Clinical and patient-related factors were collected similarly to Study I. The primary tumor site was also included in the parameters. The presenting symptoms were categorized as follows: 1) neck mass, 2) pain (any sort of pain

in the otorhinolaryngological site such as sore throat, odynophagia and otalgia), 3) dysphagia/globus, and 4) other (e.g. bleeding, weight loss and asymptomatic). The presenting symptom was defined as the first symptom related to OPSCC diagnosis described to the healthcare professional either at primary healthcare or at the Department of Otorhinolaryngology - Head and Neck Surgery at the Helsinki University Hospital. The duration of presenting symptoms was defined as the interval between the date of presenting symptom onset to the date of diagnosis. The presenting symptoms, duration of symptoms, and clinical findings were compared to p16 status and combined p16 and HPV PCR status.

8.3 Study III

We detected EBV DNA, EBER, and DNA of polyomaviruses (JCV, BKV, and SV40) from tumor tissue samples of 158 consecutive OPSCC patients in Study III. All patients were diagnosed and treated with curative intent similarly to Study IV during the same period of time. DNA presence of all oncoviruses was determined by PCR and p16 IHC was performed for all tumors. EBER was analyzed and scored from TMA blocks. A tumor was considered as HPV-positive if it showed positivity for both p16 IHC and HPV DNA by PCR. The remaining combinations of HPV DNA and p16 were assessed as HPV negative. This HPV status definition is based on the suggested algorithm by Smeets et al. (40).

The results of the different detected oncoviruses were compared with known HPV and p16 status and with clinicopathological factors. In addition, we studied the prognostic impact of all detected oncoviruses by evaluating overall survival (OS) and DFS.

8.4 Study IV

For Study IV, 169 consecutive patients diagnosed with OPSCC in a 3-year period between 2012 and 2014 were evaluated. Patients treated with palliative intent or with distant metastases at time of OPSCC diagnosis (n=23) were excluded. Patients with a second primary cancer at time of OPSCC diagnosis were also excluded (n=2). In addition, patients who underwent ND and primary tumor resection as a primary treatment modality were excluded (n=53). Altogether, 91 patients met the inclusion criteria and were treated with curative intent with or without cisplatin-based IMRT. All patients had available p16 status of the primary tumor. The patient cohort included partly the same patients as Study I and II. We aimed to study the prognostic value of pGTV, nGTV, N class, T class, and stage in patients with p16-positive and p16-negative tumors.

pGTV and nGTV were determined from pre-treatment CT scans three-dimensionally by using Varian Eclipse® RT treatment planning system versions 10.0 and 11.0 (Varian Medical Systems Inc., Palo Alto, CA, USA). Omnipaque™ injection 350 mg I/ml was used for all patients to enhance contrast in RT planning CT. Nodal involvement was determined according to generally accepted criteria and volumes were calculated in cubic centimeters (cm_3).

The endpoints of the Study IV were as follows: OS, DFS, and locoregional control (LRC).

8.5 Study V

Study V included 90 consecutive OPSCC patients with available serum samples collected prospectively at the time of OPSCC diagnosis. Patients were partly the same as in Studies I to III and were diagnosed during the same period of time. All patients were treated with curative intent, specifically surgery with or without postoperative RT/CRT or with definitive RT with or without chemotherapy. HPV status was assessed with the combined status of p16 IHC and HPV DNA PCR in concordance with Study III. We aimed to determine the serum levels and tumor immunoexpression of TIMP-1 and MMP-8 in patients with HPV-positive and HPV-negative OPSCC and to assess their association with prognosis.

TIMP-1 and MMP-8 immunopositivities were evaluated and scored both in tumor and stromal cells separately from TMA blocks. TIMP-1 and MMP-8 serum concentrations were evaluated by an enzyme-linked immunosorbent assay (ELISA) kit (GE Healthcare UK Limited, Buckinghamshire, UK) and by an immunofluorometric assay (IFMA), respectively.

The survival endpoints were OS and DFS. The association between TIMP-1 and survival and MMP-8 and survival in patients with HPV-positive and HPV-negative OPSCC was compared in univariate and multivariate Cox regression models. Tumors were distributed to HPV-positive and HPV-negative subgroups as in Study III.

8.6 Laboratory analyses

8.6.1 Tumor sample collection

FFPE OPSCC tissue samples were collected from the database of the Department of Pathology, Helsinki University Hospital.

8.6.2 DNA extraction

DNA extraction for PCR analyses was performed as previously (54, 210). DNA was extracted from 4- to 10-µm thick sections sliced from OPSCC tissue blocks. The proteins were treated with salt and placed in lysis buffer (10 mM Tris-HCl, 400 mM NaCl, and 2 mM EDTA, pH 8.2) and fragmented by protease K overnight at 37 °C. Saturated NaCl was added after digestion followed by centrifugation. DNA was treated with ethanol and placed in a microcentrifuge tube for further quantitative analyses.

8.6.3 Tissue microarray blocks

Preparation of TMA blocks was performed as in previous studies (91, 211). Tumor tissue samples were first annotated for six 1-mm tumor core punctuates. The core punctuates were removed from FFPE samples with a tissue microarrayer (Beecher Instruments, Silver Spring, MD, USA) and inserted into a new paraffin block.

8.6.4 HPV detection

8.6.4.1 p16 immunohistochemistry

p16 status was evaluated by IHC on FFPE OPSCC samples. PreTreatment module (Lab Vision Corp., UK Ltd, UK) was used to treat the prepared OPSCC samples in Tris-HCl buffer (pH 8.5) after the slides were first deparaffinized and sliced. Monoclonal mouse anti-human p16INK4a (9517 CINtec Histology Kit, MTM laboratories, Germany) served as the primary antibody for analyses. Positive and negative controls were gingival tissue and a tissue slide in diluent without primary antibody, respectively. p16 was determined positive as >70% of immunostained carcinoma cells showed positivity according to WHO recommendations (97).

8.6.4.2 HPV DNA detection by PCR

External (MY09/MY11) and internal (GP05+/bioGP06+) primers were used to determine HPV DNA genotypes. The methodology was as described previously (54). Primer sets 1 and 2 from the Multiplex HPV Genotyping Kit®

(DiaMex GmbH, Germany) were used to amplify the extracted DNA. Set 1 contains nine biotinylated forward and three reverse primers. Primer Set 2 includes primers for the amplification of a β-globin gene fragment to confirm the amount and the quality of human genomic sample. To verify the absence of contamination in the amplification reactions, a negative control without genomic DNA was used. A Multiplex HPV Genotyping Kit® (DiaMex GmbH, Germany) was used to assess the HPV genotypes. This kit detects six LR-HPV genotypes (6, 11, 42, 43, 44, and 70) and 18 HR-HPV genotypes (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82). A Luminex LX-100 analyzer (Bio-Plex 200 System, Bio-Rad Laboratories, Hercules, USA) was used to analyze labeled hybrids and a median fluorescence intensity (MFI) of a minimum of 100 beads was counted for each bead set in the sample. Only tumors consisting of the HR-HPV genotype were determined as HPV positive.

8.6.4.3 HPV DNA detection by ISH

The HPV DNA ISH method was only used for PM I in Study I. The methodology has been described in detail previously (211). Briefly, a HR-HPV probe and iVIEW Blue detection kit in a Benchmark XT series stainer (Ventana Medical Systems Inc., Tuscon, Arizona, USA) were used to detect HPV by ISH from TMA blocks. The kit detects the following HR HPV subtypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66. A tumor was regarded as HPV-positive when any of the tumor spots was positive by ISH in PM I (Study I).

8.6.4.4 HPV mRNA detection by ISH

The detection of HR-HPV E6/E7 mRNA transcripts by ISH was performed with manual RNAscope® 2.5 HD Reagent kit (Advanced Cell Diagnostics, Inc., Hayward, CA). The prepared TMA spots were incubated for 1 hour at 58°C followed by treatment with hydrogen peroxidase (RNAscope® Hydrogen Peroxide). Target retrieval was performed with RNAscope® Target Retrieval Reagents and the sections were treated with protease hydridization (RNAscope® Protease Plus) for 30 minutes at 40°C. The preparation was followed by hybridization for 2 hours at 40°C with HR-HPV probe (RNAscope®) for the following genotypes: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82. The chromogenic signal was detected by diaminobenzidine (DAB) and the slides were treated with hematoxylin after the amplifiers were hydridized. A probe for an endogenous housekeeping gene peptidylpropyl isomerase B (HS-PPIB) (RNAscope®) was used as a positive control. The bacterial gene dihybrodipicolinate reductase (DapB) (RNAscope®) was used as a negative control. The staining results of the TMA spots were determined by a standard scoring system. One to three brown dots or more was considered as a positive staining result. Scoring was performed

separately by two researchers (Jaana Hagström and Reija Randén-Brady). Ambiguous cases were re-evaluated to reach an unanimous result.

8.6.5 Detection of EBV and polyomaviruses

8.6.5.1 Detection of EBV DNA

PCR and Luminex xMAP-based methods were used to detect EBV DNA. The Luminex probe gene target sequence was 5'-GGA-AAC-CAG-GGA-GGC-AAA-TCT-A-3'. The forward primer for the gene target sequence was 5'-GAC-TGT-GTG-CAG-CTT-TGA-CGA-T-3 and the reverse primer was 5'-CAG-CCC-CTT-CCA-CCA-TAG-GT-3'. The same methodology has been used in previous studies (56, 212).

8.6.5.2 Immunostaining and immunoscoring of EBER

In Study III, we used the EBER peptid nucleid acid (PNA) Probe/Fluorescein and PNA-ISH Detection Kit (Dako, Glostrup, Denmark) to detect EBERs (EBER1 and EBER 2) from TMA slides. The same methodology has been used in a previous study (56). The TMA slides were treated with Eosin and Aquamount (Dako) after incubation. We evaluated the tumor and stromal cells from TMA slides separately by two researchers (Reija-Randén Brady and Jaana Hagström). Ambiguous cases were re-scored. The scoring results of EBER were assessed as follows: negative (-), mild positivity (<20 positive cells [+]), moderate positivity (20-60 positive cells [++]), and strong positivity (>60 positive cells [+++]).

8.6.5.3 Detection of polyomaviruses

DNA of polyomaviruses (JCV, BKV, and SV40) was detected by quantitative (q)PCR (Roche, Light Cycler 96, (Roche Diagnostics, Roche Molecular Diagnostics, Pleasanton, CA, UA) in Study III. The T antigens of all polyomaviruses were amplificated by different primers. The primers for JCV were JCTAGP1 5′-TGA GGA ATG CAT GCA GAT CTA C-3′ and JCTAGP2 5′-TTT GCA GGG CAT TTT GTT TTT TAC-3′. The primers for BKV were BKVTAG1 5′-TTG ACT AAG AAA CTG GTG TAG ATC-3′ and BKVTAG2 5′-AGA GTG GGA GTC CTG GTG GAG TTC C-3′. The primers for SV40 were SVTAGP1 5′-TTA GCA ATT CTG AAG GAA AGT CCT TG-3′ and SVTAGP2 5′-AGC AGT GGT GGA ATG CCT TTC ATG AGG-3′. The qPCR methodology has been described previously (54, 213).

8.6.6 Methodology of TIMP-1 and MMP-8

8.6.6.1 Immunohistochemistry and immunological methods

Immunohistochemical staining of TIMP-1 and MMP-8 were analyzed from TMA blocks. Monoclonal mouse IgG_{2B} (R&D Systems, MAB970, Minneapolis, USA) was used as a primary antibody for TIMP-1 analysis. For immunohistochemical analysis of MMP-8, a specific polyclonal rabbit anti-human MMP-8 was used as the primary antibody (214, 215). For both TIMP-1 and MMP-8, a slide in diluent without primary antibody and gingival tissue were used as negative and positive controls, respectively.

Tumor and stromal cells in the TMA slides were scored separately by two researchers (Timo Carpén and Jaana Hagström). The scoring of MMP-8 and TIMP-1 in tumor tissue and in the inflammatory cells were evaluated according to the number of positive cells as follows: negative (0), 1-20 positive cells (1), 20-100 positive cells (2), and >100 positive cells (3) per TMA spot.

8.6.6.2 Serum analyses

Blood samples were collected prospectively and then followed by serum extraction by centrifugation at 1600 g for 10 minutes. After centrifugation, sera were stored at -70°C. IFMA was used to determine MMP-8 serum levels and an ELISA kit (GE Healthcare UK Limited, Buckinghamshire, UK) was used to assess TIMP-1 serum levels. Serum levels are presented as pmol/l (pM).

8.7 Statistical analyses

All statistical analyses were performed with IBM SPSS Statistics 25 (IBM, Somers, IL, USA). A comparison of medians of continuous variables with categorical variables was performed using Mann-Whitney U test and Kruskal-Wallis test when suitable. The two-sample t-test was used to compare means of normally distributed continuous variables between two independent groups. Chi-squared and Fisher's exact tests were used to compare categorical data. To evaluate patient outcome in Studies III to V, OS and DFS were assessed as survival endpoints. Study IV also included LRC as an endpoint. OS was defined as the time in months from the completion of treatment to death from any cause. DFS was defined as the time in months from the completion of treatment to the first recurrence or death from any cause. LRC was defined as biopsy-proven residual tumor or recurrence locally or regionally during the follow-up after treatment completion. A Cox regression model was used to evaluate hazard ratios in the univariate and multivariate analyses. In Study V, MMP-8 and TIMP-1 serum concentrations were modified by logarithmic tranformations to avoid positive skewness. Receiver operating characteristic

(ROC) curves and Youden index (sensitivity+specificity-1) were used to determine the optimal cut-off serum level of TIMP-1 to distribute patients into two groups of better and poorer survival. Survival curves were drawn using the Kaplan Meier method. Statistical significance in Kaplan-Meier estimates were calculated with the log-rank test. A two-sided *P*-value <0.05 was assessed as the statistical level of significance in all studies.

8.8 Ethical considerations

All study designs followed the guidelines and legislation of the Declaration of Helsinki. The study design and permission were approved by the Research Ethics Board of the Hospital District of Helsinki and Uusimaa, Finland (Dnr: 51/13/03/02/2013).

Informed consent with a written signature was obtained from all patients who participated in Study V. This included permission to use primary OPSCC samples and to obtain blood samples from participants for further study purposes.

9. RESULTS

9.1 Detection of HPV by different methods in OPSCC (Study I)

In Study I, we investigated the sensitivity and specificity and the positive and negative predictive values of different HPV methods against the current suggested HPV detection method in clinical practice (i.e. p16 IHC). A total of 357 consecutive OPSCC were included covering two different patient materials (PM I and PM II). ISH for HR HPV E6/E7 mRNA showed high specificity and sensitivity to detect transcriptionally active HPV in OPSCC.

The sensitivity and specificity levels varied between all methods studied. HPV mRNA ISH had the highest sensitivity (93.4%) and an excellent specificity (92.4%) when correlated with p16 IHC (Table 5). In addition, the negative and positive predictive values for p16 IHC were excellent by mRNA ISH (Table 5). Different histological detection results are presented in Figure 4.

Table 5. Sensitivity, specificity, positive predictive value, and negative predictive value of all methods when tested against p16 IHC

	xmRNA ISH (total)	DNA ISH (PM I)	DNA PCR (PM II)
Sensitivity	93.4	86.3	83.5
Specificity	92.4	95.3	89.1
PPV	95.5	96.2	94.8
NPV	89.0	83.5	69.5

Abbreviations: NPV = negative predictive value; PM = patient material; PPV = positive predictive value. xDetection of HPV mRNA for PM I and PM II was combined. Reproduced and slightly modified with permission from Elsevier.

Fifteen (11%) of the p16-positive patients were mRNA-ISH negative whereas 10 (5%) of the mRNA ISH-positive patients were p16 negative. Two of the 15 discordant cases (p16+/mRNA ISH-) were positive when assessed by a DNA-based method. Four of the p16-/mRNA ISH+ cases were positive when assessed by a DNA-based method. The discordant cases are presented in Table 6.

Table 6. Discordant cases of p16 IHC and mRNA ISH and their HPV detection by DNA ISH/PCR

Discordant cases	**Total**	**DNA ISH+**	**DNA ISH-**	**DNA PCR+**	**DNA PCR-**
	(N)	N	N	N	N
p16+/mRNA ISH- (PM I)	9	1	8		
p16+/mRNA ISH- (PM II)	6			1	5
p16-/mRNA ISH+ (PM I)	5	2	3		
p16-/mRNA ISH+ (PM II)	5			2	3

Abbreviations: N = number of patients; PM = patient material; ISH = in situ hybridization; PCR = polymerase chain reaction. HPV DNA for PM I was detected by ISH and for PM II by PCR. Reproduced and slightly modified with permission from Elsevier.

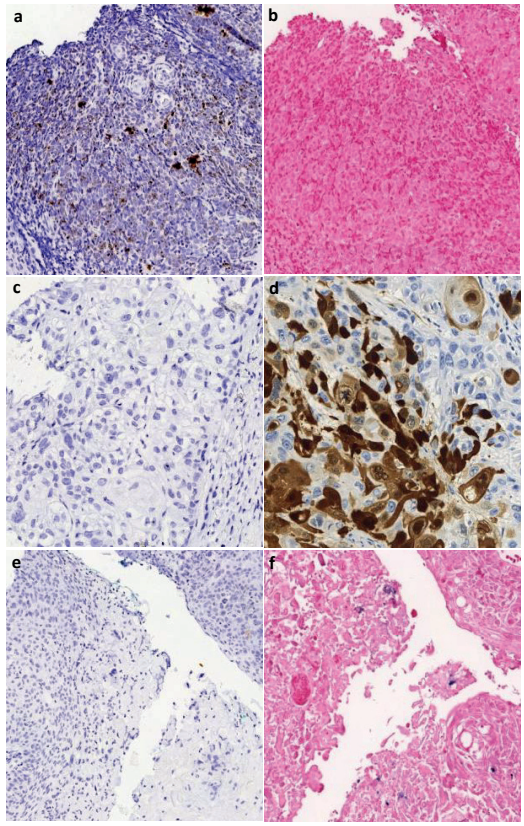

Figure 4. *A tumor sample that is HPV mRNA ISH positive (a) and p16 IHC positive but HPV DNA-ISH negative (b). Another tumor sample that is HPV mRNA ISH negative (c) and HPV PCR negative but p16 IHC positive (d). A tumor that HPV mRNA ISH negative and p16 IHC negative (e) but HPV DNA-ISH positive (f). Magnifications are x290 and x170. Reproduced and slightly modified with permission from Elsevier.*

9.2 Presenting symptoms and signs of HPV-positive and HPV-negative OPSCC and the presence of EBV and different polyomaviruses in OPSCC (Studies II-III)

Study II focused on the presenting symptoms and other clinical factors of HPV-positive and HPV-negative OPSCC patients covering a 2-year patient cohort between 2012 and 2014. Both p16 and HPV DNA status were available for 107 OPSCC samples. Two of these tumors were p16-negative but HPV DNA positive and these cases were excluded from analyses due to the small number. The remaining patients (n= 105) were categorized into three different groups according to p16 and HPV DNA tumor status as follows: HPV DNA+/p16+, HPV DNA-/p16+, and HPV DNA-/p16-.

A neck mass was the most common presenting symptom among patients with a double-positive (HPV DNA+/p16+) OPSCC. This symptom was observed significantly more often (P=0.005) when compared with patients with double-negative (HPV DNA-/p16-) OPSCC. Any sort of pain in the otorhinolaryngological area such as sore throat, odynophagia, and otalgia were the predominant initial symptoms among the majority of double-negative OPSCC patients. The discordant group (HPV DNA-/p16+) had close to parallel symptoms with the HPV double-positive group. All p16-positive tumors had significantly different presenting symptoms compared with the double-negative group. Although there was a wide diagnostic delay range in all groups, the differences between groups was insignificant. The presenting symptoms and diagnostic delay according to p16 and HPV status are presented in Table 7.

Table 7. Presenting symptoms and time delay in diagnosis of 105 OPSCC patients divided by tumor HPV DNA and p16 status

Variable	**HPV+/p16+**	**HPV-/p16+**	**HPV-/p16-**	**HPV+/p16+ vs. HPV-/p16- OR (95% CI)**	**All p16+ vs. HPV-/p16- OR (95% CI)**
N (total)	49	21	35		
Symptom					
Neck mass	26 (53.1%)	10 (47.6%)	8 (22.9%)	3.82 (1.45–10.05)*	3.68 (1.47–9.24)*
Pain	19 (38.8%)	7 (33.3%)	21 (60.0%)	n.s.	0.38 (0.16–0.87)*
Dysphagia/ globus	4 (8.2%)	3 (14.3%)	6 (17.1%)	n.s.	n.s.
Any other	3 (6.1%)	3 (14.3%)	2 (5.7%)	n.s.	n.s.
****Diagnostic delay in months**	2.8 (1–18)	2.0 (1–36)	3.0 (1–63)	n.s.	n.s.

*P<0.05, **the time from symptom onset to OPSCC diagnosis (range). Abbreviations: N=number of patients; OR = Odds ratio; n.s = nonsignificant. Reproduced and slightly modified with permission from Taylor & Francis Group.

Patient characteristics and tumor-related factors of HPV-positive and HPV-negative patients are presented more widely in Study III, which focuses on different viruses in OPSCC. This study covers a patient cohort from a 4-year time period between 2012 and 2016 and includes the majority of patients from Study II.

In Study III, the presence and prognostic role of different viruses in OPSCC (including HPV, EBV and polyomaviruses JCV, BKV, and SV40) were evaluated. A total of 158 consecutive OPSCC patients treated with curative intent were included. HR-HPV DNA was present in 97 (61.4%) tumor samples and the majority (90 samples; 92.8%) of HR genotypes were HPV16. HPV33 was present in 4 (4.1%) and HPV18 in 3 (3.1%) of the OPSCC samples. p16 was positive in 117 (74.1%) tumors and 94 were both p16 and HPV DNA positive. The double-positive patients were determined as HPV-positive (HPV+) and the remaining tumors 64 (40.5%) were determined as HPV negative (HPV-).

A total of 29.1% of samples contained BKV DNA, 20.3% EBV DNA, 13.9% JCV DNA, and only one (0.6%) tumor was positive for SV40 DNA. EBER positivity was found in 43 tumor samples (30.1%) and was present in the stromal lymphocytes close to the invasive front of tumor but not directly in the tumor cells (Figure 5). EBER positivity correlated significantly with HPV-positive tumors (P=0.026). The presence of viruses and their relation to p16 and HPV are presented in Table 8 (Carpén et al. unpublished results).

Table 8. Relation of p16 and HPV in all viruses detected

	p16+	**p16-**	***P***	**HPV+**	**HPV-**	***P****
	n (%)	n (%)		n (%)	n (%)	
HPV DNA+	94 (96.9)	3 (3.1)	**<0.001**			
HPV DNA-	23 (37.7)	38 (62.3)				
EBV DNA+	27 (84.4)	5 (15.6)	0.136	24 (75.0)	8 (25.0)	**0.045**
EBV DNA-	90 (71.4)	36 (28.6)		70 (55.6)	56 (44.5)	
EBER+	36 (83.7)	7 (16.3)	0.080	32 (74.4)	11 (25.6)	**0.026**
EBER-	69 (69.7)	30 (30.3)		54 (54.5)	45 (45.5)	
JCV DNA+	18 (81.8)	4 (18.2)	0.370	15 (68.2)	7 (31.8)	0.371
JCV DNA-	99 (72.8)	37 (27.2)		79 (58.1)	57 (41.9)	
BKV DNA+	34 (73.9)	12 (26.1)	0.980	27 (58.7)	19 (41.3)	0.896
BKV DNA-	83 (74.1)	29 (25.9)		67 (59.8)	45 (40.2)	
SV40 DNA+	0 (0.0)	1 (100.0)	0.259	0 (0.0)	1 (100.0)	0.405
SV40 DNA-	117 (74.5)	40 (25.5)		94 (59.9)	63 (40.1)	

Abbreviations: n = number of patients. *P* = *P*-value. *P*-values <0.05 are bolded. *= Statistical significance when HPV DNA+/p16+ group is compared with a group of other combinations of HPV DNA and p16. Reproduced and slightly modified from the original version that has been submitted (Carpén et al. unpublished results).

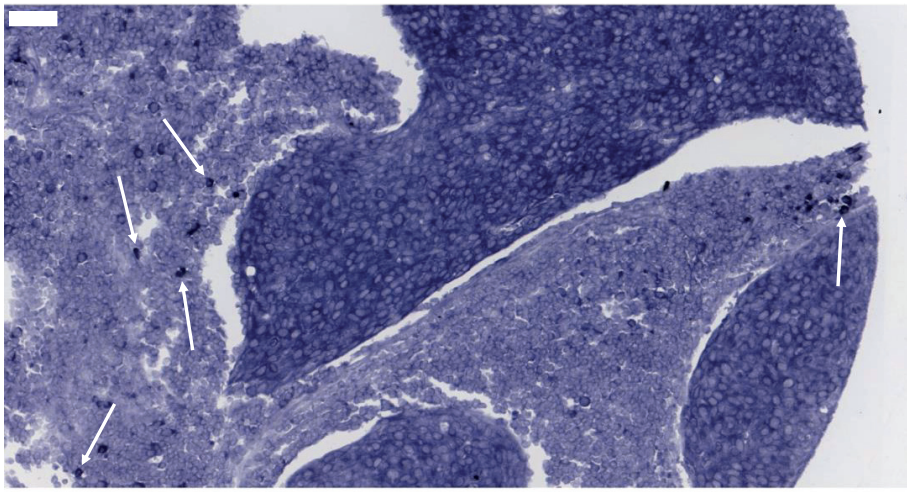

Figure 5. *Positive EBV-encoded small RNA expression in the stromal lymphocytes close to the tumor invasive front (white narrows). Scale bar length and magnification were 50 µm and x250, respectively. Reproduced and slightly modified from the original version that has been submitted (Carpén et al. unpublished results).*

HPV was the only virus that distinctly stratified patients into two disease entities by clinicopathological factors. Male sex was predominant in both groups but significantly more common among HPV-positive patients compared with HPV-negative patients. Tobacco smoking and heavy alcohol use were significantly more common among HPV-negative patients compared with HPV-positive OPSCC patients. However, the majority of HPV-positive patients were also ex- or current smokers. HPV-positive OPSCC patients often presented with disease extension to cervical lymph nodes at the time of diagnosis and the most common tumor site was tonsil. In addition, tumor grade was significantly higher and clinical stage lower according to the 8th edition when compared with HPV-negative OPSCC. EBER positivity was characteristic for smaller primary tumors (T1-T2) and for tonsil tumors. The patient and tumor-related factors according to HPV and EBER status are presented in Table 9 (Carpén et al. unpublished results). Neither JCV-, BKV-, nor SV40-positive tumors exhibited significant differences in clinicopathological factors when compared with the negative tumors (data not shown).

Table 9. Demographic and tumor-related factors according to HPV and EBER status (Study III)

Variable	HPV+*	HPV-**	*P*	EBER+	EBER-	*P*
N	94 (59.5)	64 (40.5)		43 (30.3)	99 (69.7)	
Mean age	60.8	62.1	0.400	62.5	61.0	0.360
Sex			**0.033**			0.663
Male	77 (81.9)	43 (67.2)		32 (74.4)	77 (77.8)	
Female	17 (18.1)	21 (32.8)		11 (25.6)	22 (22.2)	
Smoking			**<0.001**			0.723
Non-smoker	33 (35.1)	10 (15.6)		13 (30.2)	27 (27.3)	
Ex-smoker	39 (41.5)	10 (15.6)		14 (32.6)	28 (28.3)	
Current smoker	22 (23.4)	44 (68.8)		16 (37.2)	44 (44.4)	
Heavy alcohol use			**0.001**			0.419
None	52 (72.2)	24 (40.0)		17 (56.7)	51 (57.3)	
Former	6 (8.3)	13 (21.7)		6 (20.0)	10 (11.2)	
Current	13 (19.4)	23 (38.8)		7 (23.3)	28 (31.5)	
T class			0.925			**0.049**
T1-T2	61 (64.9)	42 (65.6)		33 (76.7)	59 (59.6)	
T3-T4	33 (35.1)	22 (34.4)		10 (23.3)	40 (40.4)	
N class			**0.002**			0.087
N0	9 (9.6)	18 (28.1)		4 (9.3)	21 (21.2)	
N+	85 (90.4)	46 (71.9)		39 (90.7)	78 (78.8)	
Stage			**<0.001**			0.069
I-II	75 (79.8)	32 (50.0)		34 (79.1)	63 (63.6)	
III-IV	19 (20.2)	32 (50.0)		9 (20.9)	36 (36.4)	
Tumor site			**<0.001**			**0.002**
Tonsil	69 (73.4)	26 (40.6)		36 (83.7)	50 (50.5)	
Base of tongue	24 (25.5)	18 (28.1)		4 (9.3)	33 (33.3)	
Soft palate	1 (1.1)	15 (23.4)		2 (4.7)	12 (12.1)	
Posterior wall	0 (0)	5 (9.5)		1 (2.3)	8 (4.0)	
Grade			**<0.001**			0.346
I	0 (0)	4 (6.3)		0 (0)	4 (4.0)	
II	7 (7.4)	21 (32.8)		6 (14.0)	19 (19.2)	
III	87 (92.6)	39 (60.9)		37 (86.0)	76 (76.8)	
Treatment			0.273			0.265
RT/CRT	61 (64.9)	36 (56.3)		24 (55.8)	65 (65.7)	
Sx +- RT/CRT	33 (35.1)	28 (43.8)		19 (44.2)	34 (34.3)	

Abbreviations: CRT = chemoradiotherapy, N+ = extend to local lymph nodes, *P* = *P*-value, RT = radiotherapy, Sx = surgery. Chi-square test was used for cross tabulation and Fisher's Exact Test when needed. *P*-values <0.05 are bolded. Percentages may not add up to 100 because of rounding. *Tumors that were p16 positive and HPV DNA positive were classified as HPV+. **The remaining HPV-DNA/p16 combinations were considered HPV-. Reproduced and slightly modified from the original version that has been submitted (Carpén et al. unpublished results).

9.3 The prognostic impact of HPV, EBV, and polyomaviruses in OPSCC (Study III)

All patients in Study III were treated with curative intent. The treatment modalities for HPV-positive and HPV-negative OPSCC and for EBER-positive and EBER-negative tumors are presented in Table 9. Patients with HPV-positive OPSCC had significantly more favorable OS (P=0.002) and DFS (P=0.001) than those with HPV-negative tumors (Figure 6). Patients with EBER+/HPV- OPSCC had a poorer outcome than those with HPV-/EBER-tumors and significantly poorer survival than those with HPV+ OPSCC regardless of EBER expression (Figure 6). The impact of polyomaviruses on prognosis was insignificant (data not shown).

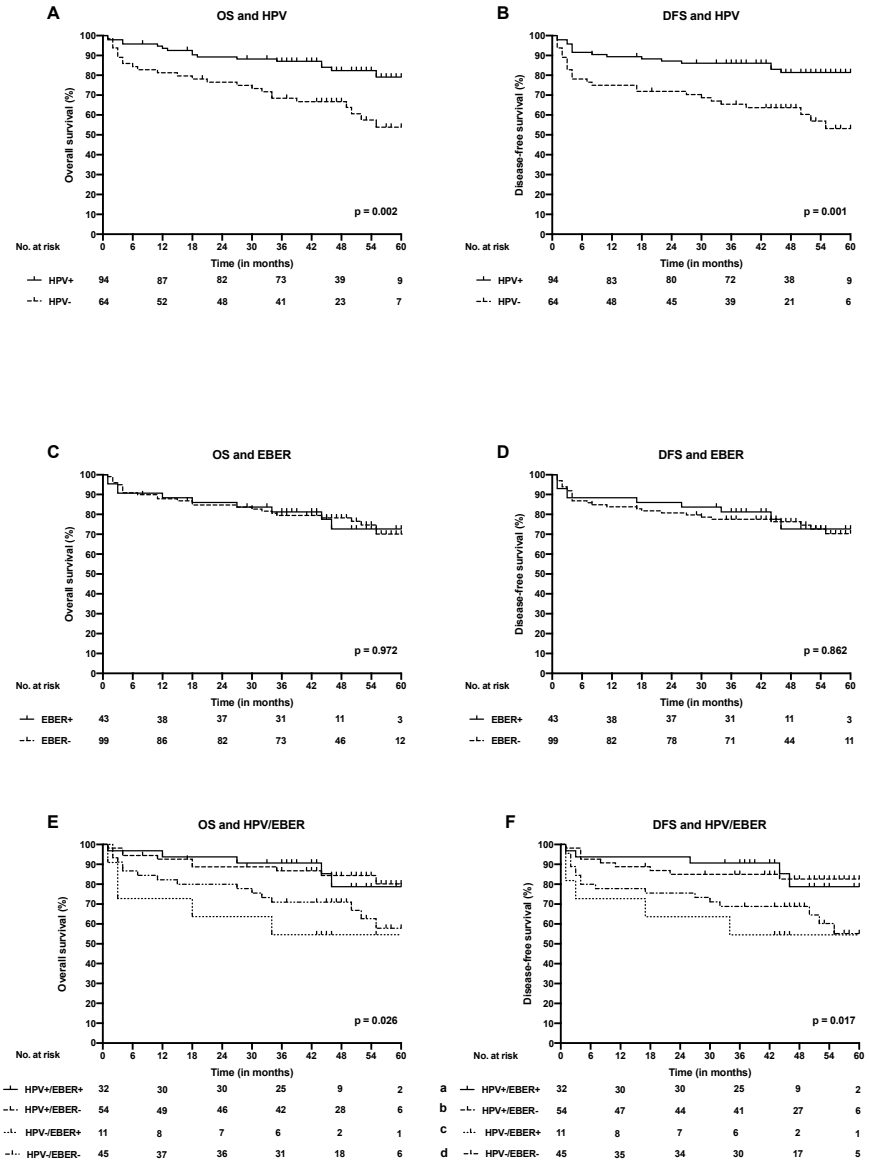

Figure 6. *Overall survival (OS) curves according to HPV and EBER in OPSCC. (A) OS of patients with HPV-positive and HPV-negative OPSCC. (B) DFS of patients with HPV-positive and HPV-negative OPSCC. (C) OS of patients with EBER-positive and EBER-negative OPSCC. (D) DFS of patients with EBER-positive and EBER-negative OPSCC. (E) OS of patients with different combinations of EBER and HPV in OPSCC . (F) DFS of patients with different combinations of EBER and HPV in OPSCC. Reproduced and slightly modified from the original version that has been submitted (Carpén et al. unpublished results).*

9.4 pGTV and nGTV as prognostic markers in p16-positive and p16-negative OPSCC patients (Study IV)

In Study IV, we examined the prognostic value of both pGTV and nGTV in 90 p16-positive and p16-negative OPSCC patients. All patients were treated with IMRT with or without cisplatin-based chemotherapy.

Higher pGTV was significantly associated (*P*=0.020) with impaired DFS in a multivariate model among p16-negative patients. nGTV showed prognostic significance when OS and LRC among both p16-positive and p16-negative patients were evaluated (Table 10). pGTV and nGTV showed a stronger association with prognosis when compared with the new (8th) edition of TNM classification and staging (Table 10).

Table 10. Multivariate analysis of tumor volume and TNM classification evaluating overall survival, disease-free survival, and locoregional control according to p16-positive and p16-negative OPSCC

	p16-positive patients			**p16-negative patients**		
	P-value	HR	95% CI	*P*-value	HR	95% CI
OVERALL SURVIVAL						
pGTV	0.336	1.0	0.99–1.02	**0.020**	1.02	1.00–1.04
nGTV	0.965	1.0	0.98–1.02	**0.027**	1.02	1.00–1.04
T class	0.857	0.9	0.27–2.99	0.096	4.27	0.77–23.57
N class	0.130	2.8	0.74–11.07	0.150	3.28	0.65–16.52
Stage	0.060	3.2	0.95–11.17	**0.046**	9.86	1.05–93.03
DISEASE-FREE SURVIVAL						
pGTV	0.364	1.0	0.99–1.02	0.106	1.01	0.99–1.03
nGTV	**0.005**	1.0	1.01–1.03	**0.022**	1.02	1.00–1.04
T class	0.825	0.8	0.30–2.59	0.368	1.85	0.49–7.03
N class	0.361	1.8	0.50–6.64	0.366	1.88	0.48–7.44
Stage	0.059	2.8	0.96–8.18	0.185	2.62	0.63–10.93
LOCOREGIONAL CONTROL						
pGTV	0.839	1.0	0.96–1.05	0.162	1.01	0.99–1.03
nGTV	**0.007**	1.0	1.01–1.05	**0.017**	1.02	1.00–1.04
T class	0.740	1.3	0.26–6.60	0.350	2.12	0.44–10.17
N class	0.805	1.3	0.15–11.42	0.117	3.63	0.73–18.19
Stage	0.290	2.3	0.47–12.14	0.139	3.82	0.650–22.58

All variables were calculated in separate multivariate models where age and gender were adjusted. pGTV and nGTV are continuous variables. T class, N class, and stage are dichotomous variables. *P*-values <0.05 are bolded. Reproduced and slightly modified with permission from Elsevier.

The mean pGTV for p16-negative patients was 38 cm_3 (SD 45.87) and the median pGTV was 19 cm_3 (range 1–147 cm_3). Among p16-negative patients, the mean nGTV was 13 cm_3 (SD 34.22). Median nGTV was indeterminate as less than half of p16-negative patients had disease extent to cervical lymph nodes. p16-negative patients with a mean pGTV >38 cm_3 had significantly poorer OS (*P*=0.005) and DFS (*P*=0.028) than p16-negative patients with a smaller pGTV (≤38 cm_3). In addition, patients with a median pGTV >19 cm_3 had less favorable (*P*=0.046) LRC than p16-negative patients with a smaller pGTV.

The mean pGTV for p16-positive tumors was 23 cm_3 (SD 29.03) and median 14 cm_3 (range 1–190cm_3). Among p16-positive patients, the mean nGTV was 26 cm_3 (SD 34.12) and the median was 15 cm_3 (range, 0–200 cm_3). DFS was significantly (*P*=0.046) poorer among p16-positive patients with mean nGTV >26 cm_3 than patients with smaller nGTV.

9.5 Association of TIMP-1 and MMP-8 with prognosis in HPV-positive and HPV-negative OPSCC patients (Study V)

Study V focused on serum levels and immunopositivity of TIMP-1 and MMP-8 in both HPV-positive and HPV-negative OPSCC patients. The association between these molecules with prognosis was assessed. The study cohort consisted of 90 consecutive OPSCC patients treated with curative intent. Serum samples were collected prospectively and all patients had available tumor tissue for immunohistochemical staining.

Although TIMP-1 immunoexpression presented as cytoplasmic positivity directly in the majority of tumor cells (84.5%), there were no significant differences in expression levels between HPV-positive and HPV-negative tumors. Immunoscoring of lymphocytes was not feasible as very few cells were positive for TIMP-1. We found no positive MMP-8 immunoexpression in tumor cells. However, MMP-8 immunoexpression was observed in most samples (91.6%) in the inflammatory leukocytes near the tumor cells. MMP-8 immunoexpression was higher among HPV-negative OPSCCs but the difference was borderline insignificant (*P*=0.052) when compared with HPV-positive tumors. No differences in TIMP-1 or MMP-8 immunoexpression were associated with survival.

TIMP-1 serum concentrations were approximately 10 times higher compared with MMP-8 serum levels both in HPV-positive and HPV-negative patients. Among HPV-positive patients, mean serum levels of MMP-8 and TIMP-1 were 761 pM (SD 743) and 8206 pM (SD 4291), respectively. In HPV-negative patients, mean serum levels of MMP-8 and TIMP-1 were 844 pM (SD 699) and 7869 pM (SD 3128), respectively. Nevertheless, TIMP-1 or MMP-8 serum

levels did not differ significantly between HPV-positive and HPV-negative patients.

MMP-8 serum levels were not associated with patient survival regardless of HPV. In addition, TIMP-1 serum levels were not prognostic among HPV-positive patients. Instead, high TIMP-1 levels in serum were independently associated with poorer OS (adjusted HR 14.7, 95% CI 1.8–117.4; *P*=0.011) and DFS (adjusted HR 8.7, 95% CI 1.3–57.1; *P*=0.024) among HPV-negative patients (Table 11).

Table 11. Multivariate analysis of overall survival according to HPV-positive and HPV-negative OPSCC patients

Variable	**HPV-positive patients**			**HPV-negative patients**		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age	1.1	0.9–1.2	0.119	1.0	0.9–1.1	0.249
Smoking			**0.016**			0.399
Ex-smoker vs. never	3.0	0.3–30.0	0.347	2.7	0.2–35.4	0.451
Current vs. never	14.5	1.7–127.6	**0.016**	3.1	0.6–16.6	0.176
Stage						
III-IV vs. I-II	1.7	0.3–10.5	0.562	8.7	1.5–50.6	**0.017**
TIMP-1 serum level	1.1	0.1–12.1	0.958	14.7	1.8–117.4	**0.011**

P-values <0.05 are bolded. Age, smoking, stage, and TIMP-1 were adjusted as all had a statistically significant association with OS in univariate analysis. Reproduced and slightly modified with permission from Springer.

We wanted to evaluate whether there is an optimal cut-off for the TIMP-1 serum level to stratify patients into groups of better and poorer survival. A TIMP-1 serum level of 7000 pM was close to the median serum level of both HPV-positive and HPV-negative patients and as a cut-off value it maximized the Youden index. Therefore, this value was chosen as an optimal serum cut-off concentration for Kaplan-Meier analyses. OS (*P*=0.006) and DFS (*P*=0.010) were significantly poorer among HPV-negative patients with high TIMP-1 serum levels (>7000 pM) than those with lower serum levels. This observation was not observed in HPV-positive patients. Survival curves drawn by Kaplan-Meier estimate are illustrated in Figure 7.

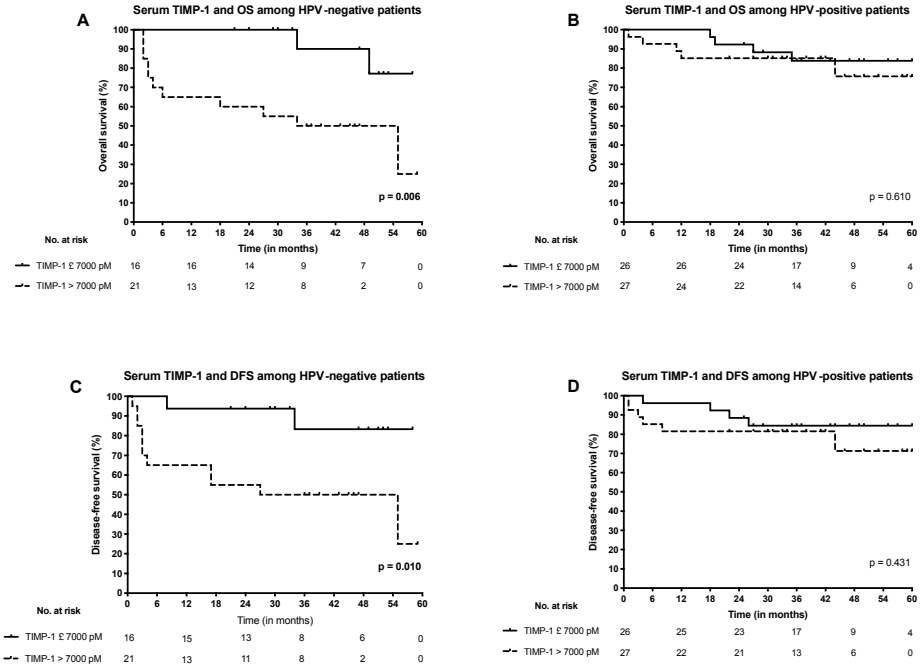

Figure 7. *Overall survival (OS) and disease-free survival (DFS) curves according to high (>7000 pM) and low (≤7000 pM) serum levels of tissue inhibitor of metalloproteinase-1 (TIMP-1) both in HPV-positive and HPV-negative OPSCC. TIMP-1 serum level and OS in HPV-negative OPSCC (A). TIMP-1 serum level and OS in HPV-positive OPSCC (B). TIMP-1 serum level and DFS in HPV-negative OPSCC (C). TIMP-1 serum level and DFS in HPV-positive OPSCC (D). Reproduced and slightly modified with permission from Springer.*

10. DISCUSSION

Interest in OPSCC has grown due to the remarkable increase in the incidence of this disease due to HPV infection. The indisputable etiologic role of HPV and association with favorable prognosis in OPSCC are well known. Accordingly, there are several ongoing trials evaluating de-escalated treatment strategies for HPV-related OPSCC. The trend in the development of personalized treatment approaches warrants precise diagnostics, including accurate HPV-detection methods to stratify HPV-positive and HPV-negative OPSCC patients. Inaccurate diagnostic tools for HPV detection may mislead the managing HN oncology teams to offer unnecessarily aggressive treatment modalities to OPSCC patients who are HPV false-negative, often leading to several iatrogenic side effects and impaired quality of life. In addition, OPSCC patients that are HPV false positive and receive insufficiently extensive treatment modalities are at high risk of an unfavorable prognosis. Correct stratification is not only essential for treatment strategies and clinical use but also for research purposes, such as epidemiologic and cell-line study setups. It is well established that HPV-positive and HPV-negative OPSCC differ in biological and clinical behavior. Nevertheless, OPSCC is typically diagnosed at an advanced stage and knowledge of patient-related factors, such as initial symptoms and signs, is scarce. In addition, the prognosis of HPV-negative OPSCC has remained poor despite improvements in treatment modalities. Reliable prognostic markers in addition to HPV are still under active research outside of clinical use. In addition, interest in other oncoviruses in addition to HPV (such as EBV and polyomaviruses) in HNSCC has grown. These other oncoviruses are suggested to work as potential cofactors. For example, the remarkable role of EBV in NPC is well recognized. However, knowledge regarding these oncoviruses in OPSCC is very limited and their prognostic role is unknown.

This study focused on the diagnostic and prognostic factors in OPSCC. We sought to enhance HPV-detection accuracy in OPSCC by comparing different methods against the standard method in clinical practice (p16 IHC). In addition, we wanted to evaluate the presenting symptoms and signs of HPV-positive and HPV-negative OPSCC patients to better understand the behavior of these two subgroups of cancer and to achieve earlier diagnosis. We investigated the presence and role of other viral factors, including EBV and polyomaviruses, in OPSCC and their association with patient outcome. Additionally, we wanted to study the potential prognostic value of tumor volume and MMP-8 and TIMP-1 in OPSCC.

10.1 High-risk E6/E7 mRNA ISH is an excellent method to detect transcriptionally active HPV in OPSCC

In Study I, we compared different HPV detection methods against p16 IHC in OPSCC and evaluated their additive impact with p16 IHC on the detection of transcriptionally active HPV in tumor tissue samples. In our series, the mRNA-ISH method had excellent specificity and high sensitivity to detect transcriptionally active HPV in OPSCC and showed superiority compared with DNA-based methods when the methods were tested against p16 IHC.

Different HPV-detection methods, including p16 IHC and PCR/ISH-based methods, have their strengths and limitations (33, 92). p16 IHC is sensitive for detecting biologically active HPV and is relatively inexpensive (36, 107). Therefore, p16 IHC is the leading HPV-detection method in clinical practice and the newest 8th edition of UICC/AJCC TNM classification stratifies OPSCC to HPV-positive and HPV-negative disease entities by p16 IHC status (38, 39). However, as a major limitation p16 IHC lacks specificity and thus may mislead diagnostics (33, 40). Our series is consistent with previous reports as mRNA ISH correlated strongly with p16 IHC in detecting active HPV (98, 216). Nevertheless, we found 15 tumors that were p16 positive but were subsequently HPV-mRNA negative.

The insufficient specificity of p16 IHC is well established in previous studies. It has been suggested that pRb inactivation and thus overexpression of p16 may be induced by other mechanisms such as gene mutations, point deletions, and even by other viruses without HPV (40, 43, 217). In clinical practice, this patient group requires special attention, particularly when considering de-escalated treatment approaches as patients with p16-positive but HPV-negative tumors have poorer survival than patients with p16+/HPV+ tumors (43, 122). The results of Study III support the probability that other viruses may also be related to pRb inactivation in the absence of HPV. We showed that some of the EBV-, JCV-, and BKV-positive tumors were also p16 positive but HPV DNA negative, even though HPV was the only virus that showed a significant association with p16 overexpression. Numerous studies have reported that even 20% of the OPSCCs may overexpress p16 protein without the presence of HPV and the specificity of p16 protein is even further reduced in other HNSCC subsites (92, 217). Hence, the presence of active HPV should be confirmed in all p16-positive cases by another, more reliable method; this is in concordance with earlier recommendations (218, 219).

HPV-DNA ISH has been established as a routine method to detect HPV infection in OPSCC samples as it has sufficient specificity and is relatively easy to perform on FFPE samples (40). However, the detected expression cannot discriminate transcriptionally active HPV infection from inactive and benign HPV infection. The method also lacks HPV sensitivity. Both of these are clear

limitations. (41, 107, 108, 220) In our series, when compared against p16, DNA ISH had poorer sensitivity than mRNA ISH. This may have been due to the insufficient amount of HPV DNA that keeps HPV unrecognizable to DNA-ISH (40, 41). PCR-based methods have shown high sensitivity but insufficient specificity to detect DNA of HR-HPV (40, 98, 220). However, regardless of the high sensitivity it is still possible that all detected HR-HPV infections are not related to the oncogenic process in tumor samples, which is a significant limitation of PCR-based methods (40, 220). In our study cohort, HPV-DNA PCR showed lower sensitivity and specificity against p16 IHC than the other HPV detection methods. When tested against p16 IHC, the sensitivity was even lower than the specificity. The absence of HPV DNA by PCR may result from insufficient DNA integrity, due to competition of reagents from various types of HPV infections with low copy numbers, or other laboratory-related factors (103, 104). In our material, two cases were negative by HPV-mRNA ISH but were positive by all other methods (p16 IHC and DNA ISH/PCR). This is consistent with previous reports as DNA-based methods have shown lower specificity when compared with mRNA ISH (98, 221). However, it is also possible that mRNA ISH leads to false HPV-negative results due to suboptimal laboratory implementation or due to loss of E6/E7 mRNA, which leads to decreased sensitivity as previously suggested (92, 220).

In our study, 10 cases appeared to be HPV-mRNA ISH positive but p16 negative. Viral transcripts of HPV detected by mRNA may present a natural amplification, and thus lead to improved sensitivity (218, 219). Regarding p16 IHC, a technically undetectable threshold and absent protein translation may result in negative staining, thus impairing its sensitivity to detect HPV (217, 222).

We showed that mRNA ISH has high sensitivity and specificity for HPV detection in OPSCC against p16 IHC when compared with DNA-based HPV detection methods. The mRNA ISH test is highly reliable as it allows visualization and detection of only viral E6/E7 transcripts, which indicate a clinically relevant oncogenic process induced by HR-HPV infection (92, 223). The HR E6/E7 mRNA ISH test has previously shown excellent sensitivity (97%) and specificity (93%) against the gold standard method (qRT-PCR mRNA) (220). The mRNA ISH test can be easily performed in a laboratory setting with either a manual or automated approach (102). A well-known limitation of p16 is that it is only a surrogate marker for HPV detection. The recent guidelines of the College of American Pathologists (CAP) recommend the use of p16 IHC to detect HPV. However, they also suggest that an additional HPV detection method should be performed in clinical trials if available to improve HPV detection accuracy (224). Although algorithms combining DNA ISH and DNA PCR-based methods with p16 IHC have been proposed to improve HPV detection, these algorithms have shown variable sensitivity and specificity levels, respectively (92, 102, 224). Thus far, the most

accurate and practical technique was introduced by Smeets et al (40), where p16 IHC is combined with a DNA PCR-based method. Consistent with previous studies (218, 219), we showed that ISH for high-risk HPV E6/E7 mRNA was a superior method to detect transcriptionally active HPV in OPSCC when compared to other HPV tests. p16-positive OPSCCs should be considered for retesting by the mRNA ISH method to confirm the presence of active HR-HPV, as p16 may be overexpressed due to different mechanisms in the absence of HR-HPV. Our findings are promising and it has been even suggested that mRNA ISH may also become a stand-alone test in the future. However, mRNA ISH must first become more available and further studies are needed to demonstrate its realibility and accuracy. (36, 220, 224)

10.2 Presenting symptoms and signs differ significantly between HPV-positive and HPV-negative OPSCC

HPV-positive and HPV-negative OPSCCs are both typically diagnosed at an advanced stage (23, 24, 29, 30). Extended disease often requires multimodality treatment strategies and leads to variable treatment outcomes, despite the fact that the prognosis for HPV-positive OPSCC is significantly better than HPV-negative OPSCC (22-24). In addition, radical treatment modalities often lead to iatrogenic side effects that impair quality of life (31, 32). Hence, the clinical behavior of OPSCC, including presenting symptoms, should be better understood to achieve earlier diagnosis and to avoid more invasive treatment approaches. In Study II, we showed that patients with HPV-positive tumors significantly more often reported neck mass as the presenting symptom than patients with HPV-negative tumors. The majority of patients with HPV-negative tumors presented any sort of pain in the otorhinolaryngolocial area as the first symptom. Patients with HPV DNA-/p16+ OPSCC tended to present with similar symptoms when compared with HPV DNA+/p16+ OPSCC patients, although slight differences were recognized. Our results are consistent with the report by McIlwain et al. (20). However, McIlwain et al. (20) used only p16 IHC to stratify tumors to HPV-positive and HPV-negative subgroups. To our knowledge, we were the first to evaluate the presenting symptoms according to both p16 IHC and HPV DNA PCR to achieve a more precise HPV definition. The recent report by Khalid et al. (81) also used both p16 IHC and HPV DNA to detect HPV; their results were consistent with our results from Study II. However, HPV DNA detected by PCR has limitations, as shown in Study I and in previous reports (33, 40). As reported in Study I, mRNA ISH for HPV detection was superior compared with DNA-based methods but unfortunately mRNA ISH was not available for all the patients in other studies in this thesis. The symptoms were in concordance with clinical findings, as in Study III OPSCC among HPV-positive patients had significantly more often already extended to cervical lymph nodes compared with HPV-negative patients at the time of diagnosis. These findings are

consistent with previous reports (20, 81). Tumor size and extension estimation by T class did not differ significantly between HPV-positive and HPV-negative patients. Previously, it was reported that HPV-positive primary OPSCC has well-defined borders and cervical lymph nodes are often cystic. In contrast, HPV-negative primary OPSCC often has undefined borders and tends to be more invasive to local structures (225). These observations may explain some differences in the presenting symptoms in the oropharynx between HPV-positive and HPV-negative patients, although a HPV-positive OPSCC is significantly associated with tumor extension to cervical lymph nodes and with a neck mass as the first symptom. In addition, HPV-positive and HPV-negative OPSCC had significant differences in primary tumor location and tumor grade, as nearly all HPV-positive tumors were located in the tonsils and base of tongue and tumor grade was significantly higher compared with HPV-negative tumors. These results are consistent with previous reports (20, 21, 68, 70). Smoking and heavy alcohol use were significantly more common among HPV-negative OPSCC patients compared with HPV-positive OPSCC patients, as also reported in previous studies (22, 64). Although HPV-positive OPSCC patients tend to be healthier and younger compared with HPV-negative OPSCC patients (63, 64), we found no difference in age between these two subgroups. Furthermore, tobacco smoking was relatively common among HPV-positive OPSCC patients, as over half were former/current smokers. A recent report by Lu et al. (30) incidated that HPV-related OPSCC is also predominant among patients >70 years. The median time delay in months from symptom onset to diagnosis was approximately 3 months; the difference between HPV-positive and HPV-negative patients was insignificant. It has been shown that diagnostic delay is not only patient-related but also healthcare related (29, 83, 226). Symptoms can often be confused with benign processes (such as tonsillitis and cervical cyst), which may mislead diagnostics and lead to delayed diagnosis (20, 29, 83, 85). We present more information about the clinical presence of these two different disease entities. Knowledge of clinical signs and symptoms of both HPV-positive and HPV-negative OPSCC is essential to achieve earlier diagnosis and thus prevent disease progression. General information should be offered and implemented not only for patients but also for healthcare professionals. In addition, the indicence of HPV-positive OPSCC is increasing among the elderly and smoking is not only associated with HPV-negative OPSCC. Therefore, clinicians should be aware of the possibility that HPV-positive OPSCC can also present among elderly patients and smokers.

10.3 Polyomaviruses are detectable in OPSCC and oncogenic EBV (EBER) may reveal a new subgroup of this malignancy

Numerous viruses have been detected in HNSCC samples. The most important oncogenic viruses are HPV in OPSCC and EBV in NPC (22, 54, 178, 179, 197). However, increasing evidence suggests that viral coinfections may have a specific impact on HNSCC (56, 100, 182). Despite the predominant role of HPV, little is known about other oncoviruses and coinfections in OPSCC. In Study III, we compared the presence and prognostic role of HPV, EBV, and the polyomaviruses BKV, JCV, and SV40 in OPSCC.

Different polyomaviruses, including BKV, JCV, and SV40, have been suggested to work as potential cofactors in cell transformation towards a malignant process and tumor progression in different cancers (54, 181). In our series, we found BKV DNA in 46 (29%) OPSCCs. This number is higher than earlier reports that investigated polyomaviruses in HNSCC, particularly in OPSCC (54, 99). JCV DNA was present in 14% of our tumor samples. This finding in OPSCC was expected, as these viruses are known to infect B lymphocytes, which are present in abundance in the oropharynx (213, 227). Coninfections were also found, as some HPV-positive tumors were also positive for BKV DNA and JCV DNA. However, in our series neither BKV nor JCV positivity exhibited any statistically significant differences in clinicopathological factors when compared with BKV-negative and JCV-negative tumors, respectively. In addition, the presence of BKV or JCV had no statistically significant impact on prognosis. SV40 DNA was found only in one OPSCC sample, thus further statistical analyses were not possible. SV40 DNA has been detected previously in different HNSCCs, such as in larynx cancer, but not to our knowledge in OPSCC (54). The role of polyomaviruses in OPSCC seems to be limited even though they are detectable.

In Study III, EBER expression was positive in approximately 30% of OPSCC samples. EBER expression was not found directly in the tumor but rather in the stromal inflammatory cells close to the tumor invasive front. In contrast to polyomaviruses, EBER expression significantly correlated with tumor HPV positivity. Positive EBER expression in NPC and other HNSCCs (including some OPSCC samples) has been previously reported (56, 178). In addition, although the presence of HPV/EBV coinfection in OPSCC has been shown in a previous report (182), the presence of HPV/EBV coinfection was not statistically significant. To our knowledge, we are the first to show a significant association between EBER positivity and HPV positivity in OPSCC in a relatively large patient cohort. Recently, it has been shown that oncogenic/latent EBV (i.e. EBER) may spread from EBV-infected cells and are recognized by toll-like receptor 3 that induces a intracellular cascade. This cascade may lead to induction of type-I IFNs and inflammatory cytokines and subsequent immune activation (184, 228). Thus, monocytes carrying latent EBV infection might invade the invasive tumor front to aid immune activation.

In contrast, EBV-positive mononuclear cells could pave the way for invasion of HPV-induced carcinomas (56). The presence of EBV may enhance the invasiveness of HPV-positive OPSCC tumors (182). On the other hand, the presence of EBV is related more to precancerous changes in cell surface such as epithelial dysplasia than to a higher stage or disease extension (229). In our study, EBER positivity was associated with lower T class and EBV DNA positivity with lower stage. Of note, the majority of EBER and EBV DNA-postive tumors were also HPV positive, which may explain the results. HPV-positive tumors typically present with lower T class and are classified at a lower stage (I-II) according to the newest TNM classification (38).

Neither EBER nor EBV DNA positivity was solely related to differences in patient outcome when compared with patients with EBER-negative or EBV-negative tumors, respectively. However, it appeared that among the HPV-negative subgroup, those with EBER-positive tumors had a significantly poorer outcome than patients with HPV-positive tumors. To our knowledge, this observation has not been published previously in OPSCC. With regards to EBV and HPV synergy, it is possible that HPV may inhibit lytic replication of EBV and thus contribute to induction of EBV latency (194). A recent study revealed that EBER positivity is associated with poorer survival in HNSCC (56), although EBER positivity is associated with a more favorable prognosis among NPC (178).

HPV was the most prevalent virus detected. Over half of the samples were positive for HPV and over 90% of the HPV-positive samples were HR-HPV genotype 16. These results are consistent with previous reports (22, 25). HPV was the only virus that clearly stratified patients into two different disease entities by clinicopathological factors, as discussed under the presenting symptoms and signs. In addition, we also showed significantly improved outcomes among patients with HPV-positive tumors than those with HPV-negative tumors. Our results are consistent with multiple previous reports showing the indisputable role of HPV in OPSCC (7, 22, 23, 63, 64).

According to the results of Study III, we suggest that EBV may act as a co-factor in HPV-related OPSCC, as previously suggested (182, 194). In addition, we showed that EBER analysis might identify a new subgroup of OPSCC patients with a different prognosis and it seems that HPV is not the only virus involved in OPSCC. This novel observation should be further assessed and repeated in a larger study cohort with a prospective study design.

10.4 Large pGTV and nGTV are associated with poor prognosis in OPSCC

As shown in Study III and reported in numerous previous studies, HPV clearly stratifies OPSCC into two different disease entities by behavior and prognosis (7, 22, 23, 63, 64). HPV-positive patients typically have excellent prognosis compared with HPV-negative patients. The prognosis of HPV-negative OPSCC has remained poor despite improvements in treatment strategies. In addition, there is still a subgroup of HPV-positive OPSCC patients that do not respond appropriately to current treatment. In Studies IV and V we wanted to study the utility of tumor volume and TIMP-1 and MMP-8 as potential prognostic factors among HPV-positive and HPV-negative OPSCC patients.

In Study IV, we showed new evidence of the prognostic potential of pGTV and nGTV among HPV-positive and HPV-negative OPSCC patients treated with definitive IMRT with or without cisplatin-based chemotherapy. Among p16-negative patients, higher pGTV was significantly associated with poorer OS in multivariate analysis. Large p16-negative tumors (pGTV >38 cm_3) were associated with significantly poorer OS and DFS compared with smaller tumors (≤38cm_3). In addition, p16-negative tumors of median pGTV >19 cm_3 were associated with poorer LRC compared with smaller tumors. For comparison, the difference between high-risk and low-risk groups of T category, N category, and stage on all endpoints remained insignificant among p16-negative patients. In multivariate analyses, only T category had prognostic impact among p16-negative patients, as a higher T category was associated with poorer OS. In contrast, pGTV, N category, T category, and stage had no significant impact on any of the endpoints in p16-positive patients in multivariate analyses. Our findings on the prognostic value of pGTV in OPSCC are promising and consistent with previous studies (44, 45, 48, 152, 155). To our knowledge, this is the first report to compare the association of pGTV with prognosis among both p16-positive and p16-negative OPSCC patients and according to the recently updated (8_{th} edition) TNM staging (38).

nGTV of p16-negative patients was significantly associated with outcome in all endpoints by multivariate analyses. However, significant differences were not observed between large and small nGTV groups when dichotomized by mean or median value. Therefore, although measurement of nGTV may offer prognostic estimation for p16-negative OPSCC patients, clear cut-off values remain unknown. In contrast, Lok et al. (44) did not find a significant association between nGTV and treatment outcome among OPSCC patients. However, HPV status was unknown and they used only dichotomized volumes in univariate analysis, which are clear limitations. Instead, in a previous report by Chao et al. (48) revealed that larger nGTV was significantly associated with poorer DFS and LRC in OPSCC, but as a limitation they did not stratify patients into two groups by HPV status.

Among p16-positive OPSCC patients, we found that larger nGTV was independently associated with poorer DFS and LRC, whereas a similar association was not found with the newest (8th edition) of TNM classification and staging. In addition, patients with mean nGTV >26 cm_3 had poorer DFS compared with smaller nGTVs. While these results are consistent with a report by Davis et al. (152), we were the first to show potential cut-off values in both p16-positive and p16-negative OPSCC patients.

As a limitation of the TNM classification, the definition of T and N categories are based on diametric and anatomic criteria (153, 154). In contrast, volume is measured in 3D, which seems to yield a more accurate dimensional definition of the tumor and may thus explain our results. In addition, pGTV and nGTV were used as continuous variables in multivariate analysis, which is more reliable compared with a dichotomous definition. It has been suggested that larger tumor mass (volume) may contain a greater number of malignant tumor cells (148-150), which may decrease treatment response to definitive IMRT with or without concurrent chemotherapy. HPV-positive tumors, which are often also p16-positive according to Study I and to previous studies (33, 40), differ significantly from HPV-negative tumors regarding biological and genetic backgrounds (11, 230). Our data showed also differences in tumor volumes, T classes and N classes between these two cancer subgroups. This may partly explain the differences in pGTV and nGTV regarding treatment response between patients with HPV-positive and HPV-negative tumors.

In the present study, HPV status was determined only according to p16 status. This is a clear limitation as also indicated in Study I. As a strength, the TNM classification of the tumors was assessed according to the newest (8th) edition. In addition, we used pGTV and nGTV as continuous variables in the multivariate analysis and the cut-off values were assessed by both mean and median values for Kaplan-Meier analyses.

In conclusion, pGTV may be an independent prognostic factor in p16-negative patients to estimate treatment response to definitive CRT. nGTV may serve as an independent prognostic factor for both p16-positive and p16-negative OPSCC patients. Current treatment strategies of OPSCC are moving towards personalized approaches. The main goal is to reduce iatrogenic side effects, particularly by de-escalating radiation to nearby normal tissue surrounding the tumor and by maintaining a favorable patient outcome by targeting effective doses to primary locoregional tumor areas (231). Accordingly, there are several ongoing trials evaluating treatment de-escalation approaches for HPV-positive OPSCC (43, 95, 122). Our findings may offer information for more accurate tumor definition and prognostication in future OPSCC studies, including those with larger patient cohorts and prospective designs.

10.5 Elevated serum levels of TIMP-1 are associated with poor prognosis in HPV-negative OPSCC patients

In Study V, we evaluated the prognostic value of MMP-8 and TIMP-1 in serum and in tumor tissue. We found that TIMP-1 levels in serum may have prognostic significance for patients with HPV-negative OPSCC and may work as an independent prognostic biomarker for this subgroup of cancer. After other covariates were adjusted, elevated TIMP-1 serum levels were independently associated with poorer OS and DFS among HPV-negative OPSCC patients. TIMP-1 serum levels have shown prognostic significance in other cancers (51, 172, 173), but to our knowledge the present report is the first to show its significance in OPSCC. In addition, our study design was the first to compare the prognostic value of TIMP-1 and MMP-8 both in HPV-positive and HPV-negative OPSCC patients.

The function of TIMP-1 is complex as it has been reported to have two distinct cell-surface functions. TIMP-1 has a specific inhibitory function but also works as a growth factor (170, 174, 175). It directly regulates various MMPs but also interacts with the cell surface molecule CD63. This leads to activation of an intracellular signaling pathway through FAK, resulting in cell proliferation (158, 159, 174, 175). Although TIMP-1 inhibits and regulates MMP-8 function, MMP-8 serum levels were not associated with prognosis in Study V. MMP-8 is associated with a more favorable prognosis in breast cancer and tongue cancer (165, 166), whereas other MMPs are associated with tumor invasiveness and impaired prognosis in several cancers (49, 162, 163).

According to our findings, the elevated TIMP-1 serum levels associated with unfavorable prognosis without the prognostic impact of MMP-8 raises the possibility that the association between TIMP-1 serum concentration and survival is not directly mediated by inhibition of MMPs, but instead by FAK activation due to interactions with CD63. FAK has a crucial role at the cell surface by regulating immunoevasion and tumor growth and has also been proposed as a possible target for immunotherapy (157, 232, 233).

In our study, the association between elevated TIMP-1 serum levels and poorer prognosis among HPV-negative OPSCC patients was evident. The prognostic association of increased TIMP-1 serum levels and survival remained statistically insignificant among HPV-positive OPSCC patients. It is possible that TIMP-1 upregulation does not have a specific and sufficient function in oncogenic changes in HPV-positive OPSCC. Instead, the oncogenic changes leading to TIMP-1 upregulation may be responsible for the poorer prognosis observed with HPV-negative tumors. Another important aspect is that on average HPV-negative OPSCC have more oncogenic mutations and the mutation profile is distinct when compared with HPV-positive OPSCC (13, 14,

16), which may lead to different cell surface functions. In addition, in the present study HPV-positive patients had a relatively small number of events that may lead to insignificant results.

TIMP-1 immunopositivity was present in the majority of tumor samples. However, we did not find an association between TIMP-1 immunoexpression and prognosis, in contrast to previous studies (169, 170, 177). One explanation for this may be that TIMP-1 is secreted early from OPSCC tumor cells and that tissue immunoexpression does not directly indicate the production rate of TIMP-1 in OPSCC cells. MMP-8 expression was not found directly in the tumor, but instead in the adjacent inflammatory cells. While the level of MMP-8 expression tends to be higher among HPV-negative OPSCC patients than those with HPV-positive OPSCC, the difference did not reach statistical significance. In addition, the association between different MMP-8 immunoexpression levels and prognosis was insignificant.

The survival rates of HPV-negative OPSCC patients have remained generally poor despite developments in treatment strategies (7, 22, 24). Hence, there is a clear demand for new prognostic markers, particularly for HPV-negative patients. Study V provides new evidence for the potential of TIMP-1 serum levels to serve as an independent prognostic biomarker for this subgroup of cancer. This observation should be re-evaluated and studied in a larger cohort in a multi-center setting with a prospective design.

10.6 Study strengths and limitations

Clinical and tumor-related factors were available for all studies and were collected systematically. In Studies III to V, the follow-up period was relatively long, which is a clear strength. Studies I and III had a relatively large number of patients. OPSCC samples were available for all viruses detected by PCR in Study III. In Study IV we used the newest (8th) edition of the TNM classification, which yielded more extensive information on the prognostic significance of the TNM classification than the 7th edition. In addition, all serum samples were collected prospectively at OPSCC diagnosis in Study V, which is another clear strength of the study setup.

As limitations in all studies, the information of the alcohol use was not available for each patient and HPV methodology varied between different studies. In Study I, DNA-based HPV-detection methods were different between PM I and PM II and parallel analyses were not feasible due to the relatively small amount of sample material in the TMA spots. We did not have fresh-frozen samples, and thus analysis of viral HPV E6/E7 mRNA transcripts by qRT-PCR in tumor tissue was not possible. In addition, immunohistochemical staining of EBER, MMP-8, and TIMP-1 were not

available for each OPSCC sample due to insufficient tumor size or absence of tumor tissue. The study design was retrospective in all studies, except for blood sample collection that was performed prospectively in Study V. Studies IV and V had a small number of patients and the overall number of events was relatively small; more comprehensive statistical analyses were therefore not possible.

10.7 Concluding remarks and future prospects

The increasing incidence of OPSCC and the significant role of HPV in this malignancy are evident. OPSCC is still typically diagnosed at an advanced stage and thus often requires multimodality treatment approaches to achieve favorable patient outcome. In addition, the detection of HPV in tumor samples has remained inaccurate in routine diagnostics. We have shown new evidence on the potential of mRNA ISH to be a reliable and precise diagnostic tool to detect transcriptionally active HPV in OPSCC. The HPV detection method should be highly reliable to stratify tumors accordingly, as treatment development is evolving towards highly personalized approaches. In addition, we showed significant differences in presenting symptoms and other clinical findings between HPV-positive and HPV-negative patients. These findings provide a new understanding of these two cancer subgroups and may presumably facilitate earlier diagnosis. Our observations support the inundisputable role of HPV in OPSCC but also offer novel findings on EBV. The role of EBV, particularly EBER, should be considered in future study designs as it may act as a cofactor and have prognostic impact on OPSCC. Tumor volume and TIMP-1 serum level showed significant prognostic values, particularly among HPV-negative OPSCC patients. New prognostic markers are necessary to achieve better patient outcomes and to guide personalized treatment in the future. While the development of new diagnostic and prognostic tools are essential for OPSCC, the most important goals are HPV prevention by vaccination and policies against tobacco smoking and heavy alcohol use.

11. CONCLUSIONS

- We assessed the methodology to detect transcriptionally active HPV in OPSCC. We observed that HPV mRNA ISH showed superiority compared with other HPV detection methods. We suggest that all p16-positive tumors should be retested by HR HPV E6/E7 mRNA ISH, as the p16 protein may be overexpressed due to HPV-independent mechanisms. Accurate HPV methodology is not only important in estimating clinical outcomes and diagnostics but also in future study designs that focus on more personalized treatment strategies.

- We further clarified the clinical presentation of HPV-positive and HPV-negative OPSCC. Our findings provide additional information regarding the patient-related factors of these two cancer subgroups. Awareness of the symptoms and signs of these two cancer subgroups is essential for patients and healthcare professionals to achieve a correct diagnosis as early as possible, which may improve patient outcomes and avoid reduction in quality of life.

- We showed that polyomaviruses are detectable in OPSCC but seem to have no association with clinicopathological features or prognosis. EBER expression was not found in tumor cells but instead in the stromal lymphocytes adjacent to the invasive front of tumor. EBER expression also correlated significantly with HPV positivity. HPV was the only virus that had a significant impact on prognosis and clearly distributed OPSCC into two disease entities according to clinicopathological factors. However, EBER expression in HPV-negative OPSCC was associated with poor survival, which to our knowledge is a novel observation. Hence, EBER analysis may identify a new OPSCC subgroup that is not related to HPV and this should be considered in future study setups.

- We observed that higher nGTV was significantly associated with poorer outcome both in p16-positive and p16-negative OPSCC patients. In addition, higher pGTV was significantly associated with poorer survival among p16-negative patients. The prognostic accuracy of pGTV and nGTV were better than the newest TNM classification. Measurement of tumor volume may therefore offer a more precise tumor definition and prognostication in OPSCC. Accurate tumor definition and new prognostic factors are necessary as treatment design is evolving towards more personalized approaches.

- In the present study, we were the first to show a significant association between elevated TIMP-1 serum levels and survival among patients with HPV-negative OPSCC. According to our findings, TIMP-1 serum levels may serve as an independent prognostic biomarker in HPV-negative OPSCC. However, these findings should be validated and confirmed in a larger cohort in a multicenter setting.

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